TEXAS REPORTS on BIOLOGY and MEDICINE

VOLUME 12, NUMBER 4 WINTER, 1954

Copyright 1954 by the Board of Regents of The University of Texas

Contents

M. D. ANDERSON HOSPITAL EIGHTH ANNUAL CANCER SYMPOSIUM

	Page
The Bence-Jones Protein of Multiple Myeloma, *Jorge Awapara and B. Jirgensons*	799
An Evaluation of Some Comparative Studies on Cultured Strains of Normal and Malignant Cells of Animals and Man, *George O. Gey, Frederik B. Bang, and Margaret K. Gey*	805
Evidence of the Functional Differentiation of the Embryonic Chick Thyroid in Tissue Culture, *Federico Gonzales*	828
Cytological Studies on HeLa, A Strain of Human Cervical Carcinoma. I. Observations on Mitosis and Chromosomes, *T. C. Hsu*	833
Studies on Human Cancer Using Sponge Matrix Tissue Culture. I. The Growth Patterns of a Malignant Melanoma, Adenocarcinoma of the Parotid Gland, Papillary Adenocarcinoma of the Thyroid Gland, Adenocarcinoma of the Pancreas, and Epidermoid Carcinoma of the Uterine Cervix (Gey's HeLa Strain), *Joseph Leighton*	847
Studies on Human Cancer Using Sponge Matrix Tissue Culture. II. Invasion of Connective Tissue by Carcinoma (Strain HeLa), *Joseph Leighton and Ira Kline*	865
The Use of Tetrazolium Salts for Short Term Cultures of Surgical Specimens, *Peter M. Marcuse*	874
Cytologic and Biochemical Studies on the Granulocytes in Early Leukemia Among Atomic Bomb Survivors, *William C. Moloney and Robert L. Lange*	887
The Classification and Diagnosis of Human Tumors by Tissue Culture Methods, *Margaret R. Murray and Arthur Purdy Stout*	898

[Contents Continued on Next Page]

Page

Tissue Culture of Human Breast Carcinoma and Normal Breast Epithelium, *Mary Faith Orr and Barton McSwain* 916

The Macromolecular Nucleoprotein Particles of Normal Rat Liver and Azo-Dye Induced Liver Tumors, *Mary L. Petermann*..... 921

Radiation Effects in Tissue Culture, *Agnes N. Stroud and Austin M. Brues* 931

* * * * *

Distribution of Radioiodinated Anti-Organ Antibodies in the Injected Rat, *Ludwik Anigstein, Kenneth P. McConnell, Dorothy M. Whitney, Peter Pappas, Oscar W. Portman, and William Barnes* 945

Reactions of Cells in Tissue Culture to *Hemophilus Pertussis*. I. Effect of Whole Organisms on Brain Tissue and Results of Treatment With Specific and Normal Serum, *Harriet M. Felton, Aldo Gaggero, and C. M. Pomerat* 960

Psychotherapeutic Adequacy and Judgment of Personality, *H. A. Goolishian* 972

Response of Trichomonas Hominis to Carbarsone: A Case Report from Korea, *Thomas A. Haedicke and Bobby Jones* 975

Tumors of the Salivary Glands, *Joseph K. Johnson and John H. Childers* 979

Autointoxications and Processes of Ageing, *V. Korenchevsky*..... 1006

Notes on the Subfamily Rhadinopsyllinae Wagner 1930 (Siphonaptera, Hystrichopsyllidae), and Description of a New Species, *Rhadinopsylla Multidenticulatus, Harvey B. Morlan, and M. Prince* 1037

Sanguinin: Local Wound Healing Effect on Chronic Cutaneous Ulcerations, *Samchai Nilasena, Fenwick L. Watts, J. Fred Mullins and Ludwik Anigstein* 1047

Proceedings, Texas Branch of the Society of American Bacteriologists, May 28–29, 1954 1052

An Unknown Virus-like Agent Isolated from a Patient Affected With Hodgkin's Disease, *Reginald L. Reagan, Eddy D. Palmer, Edward C. Delaha, Mildred T. Stewart, and A. L. Brueckner* 1061

Electron Microscopic Studies of An Egg-Adapted Virus Isolated From a Patient With a Common Cold, *Reginald L. Reagan, Eddy D. Palmer, Edward C. Delaha, Sue R. Cook, Arthur L. Brueckner, and Helen E. Nelson* 1066

A Separable and Multipurpose Tissue Culture Chamber, *George Rose* 1074

Calling Attention To 1084

Edited at The University of Texas Medical Branch, Galveston

M. D. ANDERSON ANNUAL SYMPOSIUM ON CANCER RESEARCH

The first symposium on cancer research under the auspices of the M. D. Anderson Hospital, Houston, was held in May, 1946. At the symposium on fundamental cancer research held on March 3, 1947, at the M. D. Anderson Hospital, reports on various aspects of cancer research were given by speakers representing Rice Institute, Baylor University College of Medicine, and The University of Texas. These were reported in abstract (*Texas Reports on Biology and Medicine* 5: 367–382, 1947), and some of the articles were subsequently reported in detail.

At this session Doctor Charles M. Pomerat, Professor of Cytology and Director of the Tissue Culture Laboratory at The University of Texas Medical Branch, Galveston, presented a discussion on *Recent Advances in the Study of Malignancy With Tissue Culture Technique*. This aroused much interest.

So important have the tissue culture methods become in cancer research that the annual symposium on fundamental cancer research held in the spring of 1954 at the M. D. Anderson Hospital, Houston, was devoted entirely to this subject. The meetings appropriately were held in the magnificent new building of the M. D. Anderson Hospital, and cancer research workers from all parts of the country were in attendance.

Most of the detailed reports offered at this symposium are published herewith. There is also included a chemical report on Bence-Jones proteins in multiple myeloma, by Doctor Jorge Awapara and Doctor V. Jirgensons. In this same issue of *Texas Reports on Biology and Medicine* are to be found other discussions which might appropriately have been included in the symposium, such as the description of a multi-purpose tissue culture chamber by Doctor George Rose (page 1074), and the report by Doctor R. L. Reagan and his associates on the isolation of a virus-like agent from a case of Hodgkin's Disease (p. 1061). Unfortunately, however, the annual E. W. Bertner Lecture, given so well by Doctor Peyton Rous, of New York City, was extemporaneous, and is not available for publication.

Tissue culture methods promise to give valuable information on cancer. It is a fascinating technique and it is rapidly becoming well standardized so that results are appropriately repro-

ducible and thus capable of full validation. A comprehensive symposium on the subject, edited by Doctor Pomerat, has currently appeared (*Ann. N. Y. Acad. Sci.*, 58: 971–1326, Nov. 17, 1954).

There is a subtle sort of excitement in cancer research, and especially when its results become available with time-lapse movies of living cells. It is entirely appropriate that this emotional feeling should be expressed poetically. The cadenced verse which recently has been read at cancer societies and other meetings seems to express some of the emotional interest and hope which cancer research inspires.

Cancer Grows Wild

What is the difference in
these lethal shapes.
that makes them deathful deviates
from quiet harmony
with those around
from which they have evolved?
They seem the same.
and yet they are
insidious consumers of
the common nutriment,
harsh foes of ordered life.
cruel tissue tyrants,
whose reeking bloom
all other overgrows.

With steady prying industry,
we find this crab-like spread,
called cancer long ago,
may yield the clues
to knowledge and control
of life's evolving mysteries;
our work may show
what cancers are in cells
or in societies:
our efforts may reveal
how life may ebb or flow.

Perhaps in another few years a cancer symposium dealing with results of tissue culture studies may have many significant reports of practical clinical value to offer. It is essential, however, first to obtain and verify the facts about cancer in every way possible, so that clinical applications may proceed on a solid basis.

THE BENCE-JONES PROTEINS OF MULTIPLE MYELOMA*

JORGE AWAPARA AND B. JIRGENSONS

The term Bence-Jones protein has come to be used to describe a protein or group of proteins found in the urine of some patients with multiple myeloma; the identifying property is the unusual behavior towards heat, *i.e.*, it precipitates out of neutral or slightly acid solutions at 45°–48°C. when slowly heated but redissolves on boiling and reappears upon allowing the solution to cool. This unique property became in time a diagnostic aid but of limited value since only less than half of the patients with multiple myeloma excrete Bence-Jones protein in the urine. Even when the patients excrete what appears to be Bence-Jones proteins in the urine, the test sometimes fails to give a positive result. We are exploring the possibility of using a flocculation test which seems to have some promise. The test is based on the fact that protein solutions can be characterized by flocculation in series of mixtures of flocculating agents. Dr. Jirgensons suggested the use of prepanol-water mixtures as the flocculating agent and for our work this system works well (1953). At certain alcohol concentrations, a protein reaches maximum flocculation; the change from maximum flocculation to no flocculation is a gradual one, usually on both sides, *i.e.*, at lower and at higher alcohol concentrations. The type of curve obtained when turbidity (index of degree of flocculation) is plotted against alcohol concentration is shown in the graph.

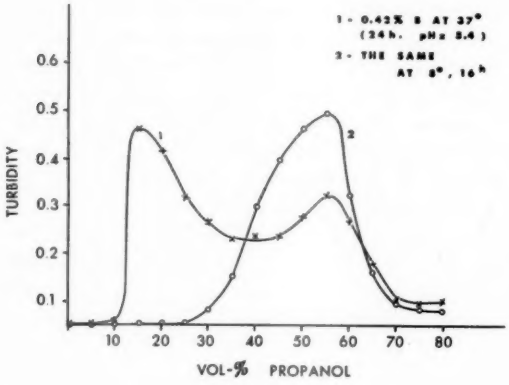
In most instances, the blood proteins of patients with multiple myeloma are in higher than normal amounts, particularly the globulins. The increase in the globulin fractions represents an increment that is not readily identifiable; in some cases one could assume that the increment is in fact a typical myeloma protein, perhaps Bence-Jones protein. But there is no acceptable

---

* From the Division of Protein Chemistry, The University of Texas, M. D. Anderson Hospital and Tumor Institute, Houston. Supported by grants from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service (C-1785) and the American Cancer Society. Presented at the Eighth Annual Cancer Symposium, April, 1954, at the M. D. Anderson Hospital, Houston.

criterion to identify a typical myeloma protein because each patient seems to produce a protein of his own. There are no properties, either physicochemical or chemical, that are common to all the myeloma proteins, regardless of source.

The problem of hyperproteinemia in multiple myeloma has been reviewed in detail by Gutman (1948). He compiled all available information up to 1941 and of the 282 cases observed, 175 or 62 per cent showed hyperproteinemia. Values as high as 14 grams of protein have been reported. The total globulin content as determined by the Howe method exceeded 3.0 grams in 65 per cent of the cases studied by Gutman *et al.* (1941), and in the same series, 15 per cent had 6.0 grams of globulins. In six instances they found values ranging from 9 to 10.4 grams.



The electrophoretic mobility of myeloma proteins varies over a wide range (Rundles, Cooper, and Willet, 1951). In some instances the increase is in the β-globulins, in others the increase is in the γ-globulin component (Kekwick, 1940). In a recently published paper, Putnam and Udin (1953) reported results from a study of 25 patients with multiple myeloma; in these series, they found a wide range of electrophoretic mobilities with a maximal incidence at the mobility of normal γ-globulins. From this investigation and others quoted earlier, it appears that the high levels of serum proteins in patients with multiple myeloma is the result of Bence-Jones proteinemia. In Gutman's words

"this suspicion is well founded. Bence-Jones proteins presumably are present in the plasma of patients with Bence-Jones proteinemia since the Bence-Jones protein must be transported by the blood from the source of origin to the kidneys." Of course, the globulin increment could still represent normal globulin, perhaps γ-globulin. Unfortunately, the γ-globulin fraction of human serum is a mixture of proteins as revealed by the difference in amino acid composition of subfraction and the difference in N-terminal amino acids in the same subfractions. The myeloma globulins vary still more; they seem to be characteristic of the patient and vary in regard to molecular weight, electrophoretic mobility, isoelectric point, N-terminal amino acid and in amino acid composition.

Much of the existent information on Bence-Jones protein is at best incomplete and difficult to assess. The problem is that proteins are difficult to identify and one is never sure whether the protein in question is a mixture or a single chemical compound. Taking into account these limitations, we have studied urinary Bence-Jones proteins both by chemical and physicochemical methods. Our results, reported here indicate that not only different patients excrete different types of Bence-Jones protein but that the same patient excretes several types of protein. Whether the various fractions obtained from the urine of one patient are different proteins or not remains a conjectural issue. We believe that the fractions are different. The evidence is what will be discussed now.

Fractionation of Urinary Bence-Jones Protein. Patient (L) had all the symptoms of multiple myeloma. The diagnosis was confirmed by X-rays and by observations on his bone marrow. This patient excreted in the urine a protein with the known properties of Bence-Jones protein. Urine was collected daily and kept in the refrigerator at all times. A 24 hour specimen was fractionated by means of ammonium sulfate. At 34 per cent saturation a protein precipitated which was removed. (Fraction DI); from the filtrate a second fraction (DII) was obtained by saturating to 43 per cent with ammonium sulfate. A third fraction (DIII) was obtained from the filtrate of DII by increasing the ammmonium sulfate concentration to 49 per cent saturation. From a second 24 hour urine specimen a fraction EI was obtained at 41 per cent saturation and from the filtrate of EI a second frac-

tion (EII) was precipitated at 49 per cent saturation. The procedures used are considered mild. In all cases, the dialyzed protein was precipitated with acetone at —5 or —10°C., and dried at reduced pressure. It is doubtful that any drastic changes would occur under these conditions.

Properties of Fractions. Four Bence-Jones protein fractions (all from the same patient) were studied in the ultracentrifuge.[1] The sedimentation revealed that the four samples studied were practically homogenous. The samples had the following S′ values at 25°C.

DI	2.99
DIII	2.80
EI	2.58
EII	2.51

Variations in sedimentation were reported by Putnam and Stelos (1953); the variations were however, in Bence-Jones proteins obtained from the urines of several patients. The values above are from specimens obtained from the urine of one patient. The amino acid composition of these fractions was determined on hydrolysates of the proteins by microbiological methods. The microbiological assay of amino acids is considered to have an error of about 8 per cent. We find it to be somewhat better than that. If 8 per cent is assumed to be the maximum error, then there are some differences in the composition of the fractions analyzed. The differences are small and therefore these results are difficult to assess. They are reported in order to show that perhaps with more accurate methods these differences could become significant. The composition of the five fractions studied is given in table 1.

Discussion

The amino acid composition of Bence-Jones proteins is not constant. Values reported by different investigators are different (Dent, and Rose 1949; Roberts, Ramasarma, and Lewis, 1950; Ågren, 1952); it is true that in each laboratory a different specimen is being studied and in the light of observations noted earlier in this paper, it would be surprising if the amino acid composition

[1] We are indebted to Mr. Tinoco and Dr. J. D. Ferry from the University of Wisconsin for carrying out these determinations.

of the proteins studied were identical. The myeloma proteins of blood have also different amino acid composition (Grisolia and Cohen, 1953). They made the observation that the amino acid composition of these proteins was similar in general terms, to the composition of γ-globulin. We made a similar observation in regards to urinary Bence-Jones protein (Awapara and Jirgen-

TABLE 1

The Amino Acid Composition of Bence-Jones Protein (L) in grams per 100 (corrected for moisture and ash)

Amino Acid	Fraction				
	DI	DII	DIII	EI	EII
Alanine	5.8	6.5	6.3	6.3	5.5
Arginine	6.1	6.9	6.4	6.4	6.3
Aspartic acid	10.8	10.1	10.6	9.6	9.8
Cystine, cysteine	4.4	2.6	3.4	2.5	3.5
Glutamic acid	17.1	18.3	14.2	18.3	17.0
Glycine	3.6	3.9	2.9	2.4	4.2
Histidine	1.0	1.1	1.5	1.1	0.8
Isoleucine	8.6	8.9	8.3	10.0	8.5
Leucine	9.1	10.2	8.9	10.6	9.0
Lysine	5.2	5.5	5.2	5.0	4.3
Methionine	0.6	1.0	0.6	0.5	1.1
Phenylalanine	5.2	5.8	4.3	5.3	5.3
Proline	4.3	7.1	4.5	6.6	5.0
Serine	11.2	10.4	10.5	10.3	9.9
Threonine	6.3	6.0	4.2	5.9	4.2
Tyrosine	5.5	5.9	5.3	5.7	5.9
Valine	10.3	8.7	9.7	8.4	9.6
Tryptophan	1.4	1.9	1.7	1.2	1.5
Total	116.5	120.8	108.5	116.1	111.4

sons, 1953). This observation cannot be taken too seriously since the errors involved in microbiological assay are relatively large. It is, however, of interest that there should be a resemblance in composition. There is the possibility that the urinary Bence-Jones protein is a breakdown product of the serum myeloma protein, which in turn could be one of the many components found in the γ-globulin increment.

REFERENCES

Ågren, G.: The Amino Acid Composition of Bence- Jones Protein. *Acta Chem. Scand.*, 6: 1232, 1952.

Awapara, J., and B. Jirgensons: Amino Acid Composition of Bence-Jones Proteins from Multiple Myeloma. 123rd. Meeting of the American Chemical Society, Los Angeles, 1953.

Dent, C. E., and G. A. Rose: The Bence-Jones Protein of Multiple Myeloma: Its Methionine Content and Its Possible Significance in Relation to the Etiology of the Disease. *Biochem. J.*, 44: 610, 1949.

Grisolia, F. T., and P. P. Cohen: Amino Acid Analysis of Serum Proteins in Multiple Myeloma. *Cancer Research*, 13: 851, 1953.

Gutman, A. B.: The Plasma Proteins in Disease. Advances in Protein Chemistry, 4: 155–250, Academic Press, 1948.

Jirgensons, B.: Characterization of Proteins by Means of Flocculation with Propanol. *Die Makromolekulare Chemie*, 10: 78, 1953.

Kekwick, R. A.: The Serum Proteins in Multiple Myelomatosis. *Biochem. J.*, 34: 1248, 1940.

Putnam, F. W., and B. Udin: Proteins in Multiple Myeloma. I. Physiocochemical Study of Serum Proteins. *J. Biol. Chem.*, 202: 727, 1953.

Putnam, F. W., and P. Stelos: Proteins in Multiple Myeloma. II. Bence-Jones Proteins. *J. Biol. Chem.*, 203: 347, 1953.

Roberts, E., G. B. Ramasarma, and H. B. Lewis: Amino Acids of Bence-Jones Protein. *Proc. Soc. Expt. Biol. Med.*, 74: 237, 1950.

Rundles, R. W., G. R. Cooper, and R. F. Willet: Relation of Abnormal Serum Components to Bence-Jones Protein in Multiple Myeloma. *J. Clin. Inv.*, 30: 1125, 1951.

AN EVALUATION OF SOME COMPARATIVE STUDIES ON CULTURED STRAINS OF NORMAL AND MALIGNANT CELLS OF ANIMALS AND MAN*

GEORGE O. GEY, FREDERIK B. BANG, AND MARGARET K. GEY

Stepping back over three decades, some of us may recall that a few of our early teachers stressed just sufficiently, and no more since they dared not be so bold, the importance of isolating, growing, and understanding the habits and character of normal and malignant cells when maintained under somewhat controllable conditions of *in vitro* growth. Some of us have long been members of this new clan and up to today have uncovered a few new facts. These are our just rewards for our sustained efforts and, now, we may say, at least, that we have fewer regrets for having continued to pursue this goal. In comparison with others in the field of experimental cancer research, we have not been as fortunate as the mouse breeding geneticists and tumor transplantation experts, since our living cell stocks cannot feed themselves without special facilities and, in addition, are most fastidious in their nutritional requirements. Furthermore, as you must realize, they tolerate only limited abuse and still require a kind of foster nurse, our tissue culture technicians, for their continued maintenance in a healthy state.

It has been almost 20 years since we first contemplated the use of an automatic culture machine which might improve continuous culture management. To my knowledge, nothing further has come of it. At least the microbiologists have achieved it with the development of the chemostat of Monod[1] and of Novick and

* From the Departments of Surgery and Parasitology, the Johns Hopkins Medical Institutions, Baltiore, Maryland. Presented at The Eighth Annual Symposium on Fundamental Cancer Research, M. D. Anderson Hospital for Cancer Research, Houston, April 5, 1954. These investigations were supported in part by grants-in-aid from the National Cancer Institute, U. S. Public Health Service, in part by the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council, in part by the National Foundation for Infantile Paralysis, and in part by the Medical Research and Development Division, Office of the Surgeon General, Department of the Army.

[1] Personal communication from Professor André Lwoff, Institut Pasteur, Paris.

Szilard. Fortunately, today, with the help of the antibiotics and improved technical skills, there are fewer limitations and good results with various cell cultures may soon be achieved, even with the animal cells growing freely in suspension, as by the technique of Gey and Shooter, developed further by Owens *et al.*

With regard to the harvesting of cells from tissues, we are not yet able to biopsy every cell system and provide it with a maintenance medium that will permit either a dormant or a proliferative existence in continuous culture. Judging from our own laboratory experiences, there are, however, many tissue and cell categories that permit a reasonable harvest of their constituent cells and which can be adapted to tissue culture for indefinite periods, and, in most cases, essentially without loss of their differentiated individualities. We have successfully grown and established many strains of cells from animals and man which have proved most useful in comparative studies of normal and malignant cells.

In this account, we have chosen to enumerate a few highlights in some of these comparative studies of cultured normal and malignant cells in the hope that it might stimulate others to appreciate some of the potentialities for fruitful research in this now very general field of experimental cell research. Many now recognize the need for more extensive applications of the many new cytologic techniques. Their efficient application will provide a better understanding of the biology of the large group of malignant diseases which basically are so poorly understood. Their ugly nature, prevalence, and persistence have caused almost every scientific discipline to be focused upon the cellular elements that constitute them.

Speaking again only from our laboratory experiences, it may be of interest to enumerate first some of the types of cells that we have maintained in continuous culture. With the exception of the normal monocyte, no normal stem blood cells have been so maintained. Regarding the maintenance of all the others in the categories enumerated, we still do not have highly selective media for the maintenance of specific cell types. Some demonstrations of those that are hormone (Gaillard) and vitamin (Fell and Mellanby) dependent or responsive have already stimulated us to carry on further research on the continuous cultivation of

cells of hormone dependent tumors and of other dependent cells, as reviewed by Furth. Some of the cell types that we have cultivated and that have responded to continuous cultivation are enumerated here and with some illustrations that may be of interest. In general, these include embryonic and extra-embryonic (placental) tissues, adult tissues, and tumorous (malignant and non-malignant) tissues. Without specific reference, the lists imply human tissues, and in many cases good growth responses apply also to many animal tissues that we have grown. It must be borne in mind that these lists are not complete and, as stated, refer particularly to the experiences of this laboratory.

1. Embryonic and extra-embryonic (placental) tissues. Continuous cultures have been established with epithelium and mesenchyme from a variety of organs. They have come from a host of tissues such as skin, muscle, thyroid, parathyroid, lung, intestine, liver, kidney, adrenal, cornea, lens, iris, pituitary, etc., and chorionic cells of several types. It should be emphasized that the epithelia are more difficult to maintain than are a whole host of fibroblastic cells or races of fibroblasts. Many tissue fragments, explanted from organs containing epithelium, will persist as organoids for very long periods of time, and only special care in the early phases of the culture management makes it possible to separate from them epithelial cells as strains due to the formative stimuli imposed by stromal elements.

2. Adult tissues. Continuous cultures of cells have been established from epithelium and mesenchyme, from the same tissues as the embryo but excluding for lack of long-term cultivation experience intestine, liver, lens, and pituitary, but including such tissues as the thymus and ovary. An example of some results with germinal epithelium of the adult mouse ovary is shown in Plate I. For example, from adult muscle, we derived strains of human fibroblasts and also maintained for long periods striated muscle cells. Pogogeff and Murray first showed it for rat and human muscle. Concerning the epithelium from the tissues listed, we have had some successful continuous culture experiences with skin, thyroid, parathyroid, lung, and kidney. These lists are given for the sole purpose of illustrating the broad cell spectrum that has given fairly good responses in our efforts to establish stable strains now proved of great value in comparative studies of normal and malignant cells.

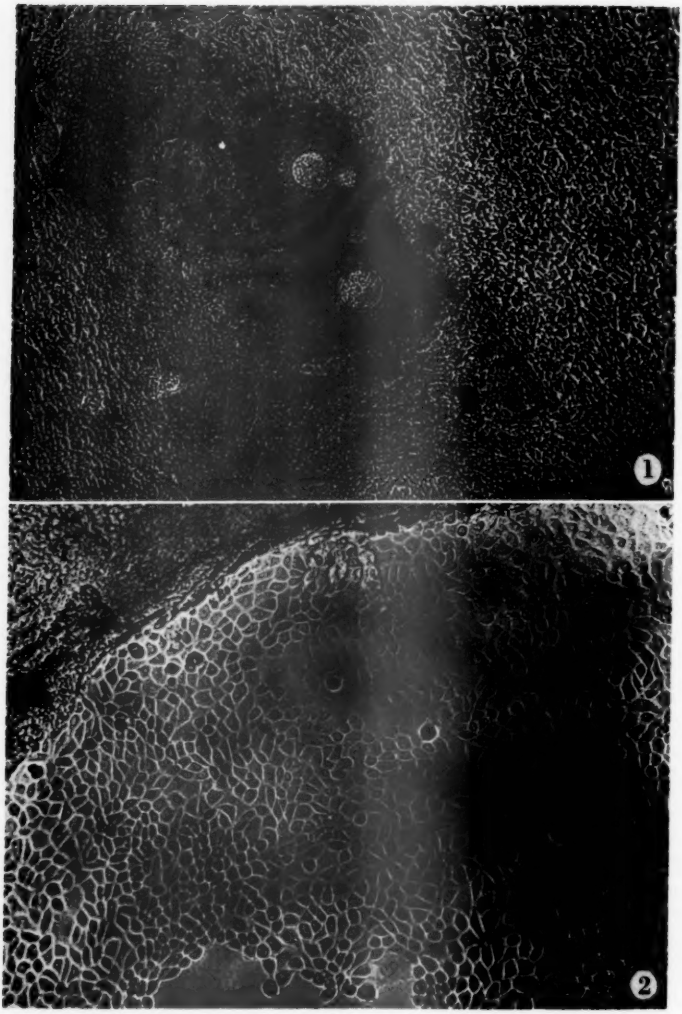


PLATE I

FIGS. 1 and 2 show membranous epithelial colonies in roller tubes from a strain of germinal epithelium of the adult mouse ovary. Figure 1 shows two clearly defined follicles which have persisted for about 6 months. Figure 2 shows a uniform epithelial spread in a 2½-year-old strain. All figures are of living cells. Mag. 120 x.

Many variations in media components for continuous cultures have been tried by various workers. A variety of mixtures containing plasma, serum, and tissue juices have been shown to be adequate. The simplest of biologic origin would still appear to be diluted serum used as fluid medium with cells growing directly on glass. Specific criteria for growth requirements remain still undefined, and broad statements are most certainly not thus far justified. Barring accidents and the effects of numerous virus and larger agents, which may pop up as contaminants, many of such cells, as referred to here, can be cultivated indefinitely. For example, fibroblastic strains or races of fibroblasts can be derived and maintained from a large variety of tissues. The fibroblastic strains *are not alike*.

3. *Tumor tissues*, from which cells can be grown indefinitely, include a host of sarcomas, such as fibrosarcoma, Ewing's sarcoma, chondroma, chondromyxosarcoma, and glioblastomas (Gey, *et al.*, 1935–54). Our oldest strain of human sarcoma (Plate II, Fig. 1) is now over 23 years old. It was explained in 1931 as was our present strain of Walker Rat Sarcoma No. 319 (Plate II, Fig. 2). Another tumor strain A.Fi., a fibrosarcoma, has been maintained over 15 years. Its excellent cultural behavior makes it most useful for cytological studies (Stoler and Gey). Many others have been grown for shorter periods. In the category of carcinomas, we have established several strains of epidermoid carcinoma of the cervix and an embryonal corcinoma of the testis. The HeLa strain (Plate III, Figs. 1–4) of epidermoid carcinoma is now over three years old, and a tissue culture recovery of a guinea pig passage (anterior chamber—eye) appears to behave like the strain of origin. Hydatid mole and, as well, chorionepithelioma should also be put into the category of strains that can be grown continuously. The recent findings of de Ruyck that a virus may be involved in the production of these tumors offers new opportunities to pursue this problem in tissue culture. There is little doubt that other tumors of man may be added to these categories of continuous culture growth potentialities should they be needed.

Fortunately, today, a variety of chemical and physical procedures for the separation of media components allows for a better understanding of the factors contributing to rapid cell growth. Many workers have concentrated their efforts on chick

cells and their response to various fractions of chick embryo extract with fewer efforts on serum fractions. Recent reports by Rosenberg and Kirk indicate that the chief growth factors in embryo extract are found in the non-dialyzable component and with some active fractions in the ultrafiltrate fraction which can be identified chemically. Their papers give a good account of the present status of this subject. Their work, however, like that of several others, does not extend into the continuous culture category.

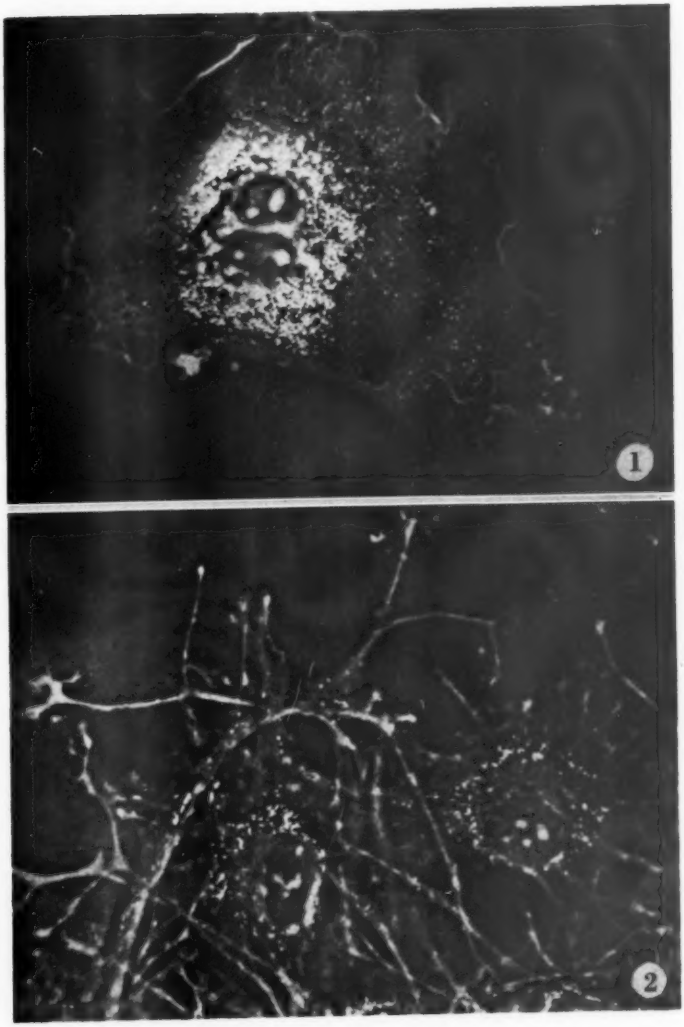
Our group has shown for some time that diluted serum alone can support growth of strains of different cell types for indefinite periods or what we have designated continuous culture growth. The best of these, human placental cord serum, used initially in our laboratory over 25 years ago, can maintain many of the cells enumerated above in continuous culture with dilutions of the cord serum from 30 to 50 per cent. Sometimes very dilute serum will maintain some cell strains for long periods. Some malignant cell strains respond much better than normal cells (Plate IV, Figs. 1 and 2) and can be grown even in diluted adult serum. Fifty per cent adult human or adult horse serum, for example, supports the growth of the HeLa strain grown directly on Pyrex glass with somewhat better results when synthetic mixtures like No. 199 of Morgan, Morton and Parker, or perhaps White's

---

PLATE II

FIG. 1 shows high dry (4 mm.) living image with *bright contrast* phase microscopy. This binucleate cell from a 23-year-old strain of human chondromyxosarcoma strain D-1 Re shows surface folds of the plasma cell membrane and numerous filamentous mitochondria throughout the cytoplasm. The broad bright mass of refractile droplets around the nuclei represent vacuolar accumulations of ingested materials which originally begin at the peripheral margins as larger dark masses which are swept to the juxtanuclear zone by protoplasmic streaming. The outermost peripheral margin, as shown to the right, may show a clear zone often free of mitochondria. It is this area which succeeds in trapping the fluid droplets (pinocytosis) shown as dark masses at the upper and lower left and which rapidly become brighter masses of smaller size. A clear account of the pinocytosis process is shown in plates V and VI from living cells under oil. Mag. 1000 x.

FIG. 2 shows two living cells, similarly photographed, of a 23-year-old strain of Walker Rat Sarcoma No. 319. Its usual form reveals many branching cytoplasmic processes rich in mitochondria and with some inclusion droplets and covered with microfibrils and microfolds. The juxtanuclear mass is usually to one side of the nucleus and shows condensed masses of mitochondria and some inclusion droplets. Mag. 600 x.


1
2

mixture are added to replace the balanced salt solution. Many modified mixtures are currently being tried out with the hope of getting improved continuous growth responses. Greatly improved growth responses are apparently now demonstrated by Healy and Parker's mixture No. 703[2] and other concoctions.

Growth of viruses in tissue culture. An ever increasing number of viruses, including new ones, are being isolated by tissue culture means. It is quite likely that the spectrum of infectious agents that can be isolated and grown will be even greater than that for the embryonated egg or animal techniques. Comparative evaluations of these techniques must await a further expansion of the techniques of tissue culture into categories more specifically concerned with a better understanding of factors affecting virus growth in or on tissue cells and the role and mechanism of of cellular immunity and antibody production at the cellular level. This most fascinating chapter cannot be written until we understand, among other things, why cells survive in the apparent presence of abundant virus. Such has been our experience, for example, with Eastern Equine Encephalomyelitis (E.E.E.) virus, a highly destructive virus when grown on a variety of normal and tumorous cells and as evaluated more recently on the HeLa cell strain. In the latter case, early destruction by E.E.E. virus of a large proportion of the cells of a HeLa cell colony is followed by rapid re-establishment of the colony by the survivors with many cells showing phagocytosis of the virus-killed and -injured cells. Even dividing cells contain phagocyted cell debris. We need to know more about such recovery phenomena.

Spectrum of virus susceptibility of the HeLa strain. Some of the viruses which may be isolated and propagated in cultures of this cell strain may be of interest to this group. They include all three types of poliomyelitis, several of the Coxsackie group, B-1, B-3, B-4, and Texas 14, Eastern and Western Equine Encephalomyelitis, West Nile, vaccinia, pseudorabies, herpes simplex, and pseudolymphocytic choriomeningitis, as shown by Scherer, Syverton and Gey, and Scherer, *et al.* Their experiences with St. Louis Encephalomyelitis and Japanese B virus (personal communication) demonstrate considerable virus production without

[2] Personal communication from Dr. Raymond Parker, Connaught Laboratory, University of Toronto, Canada.

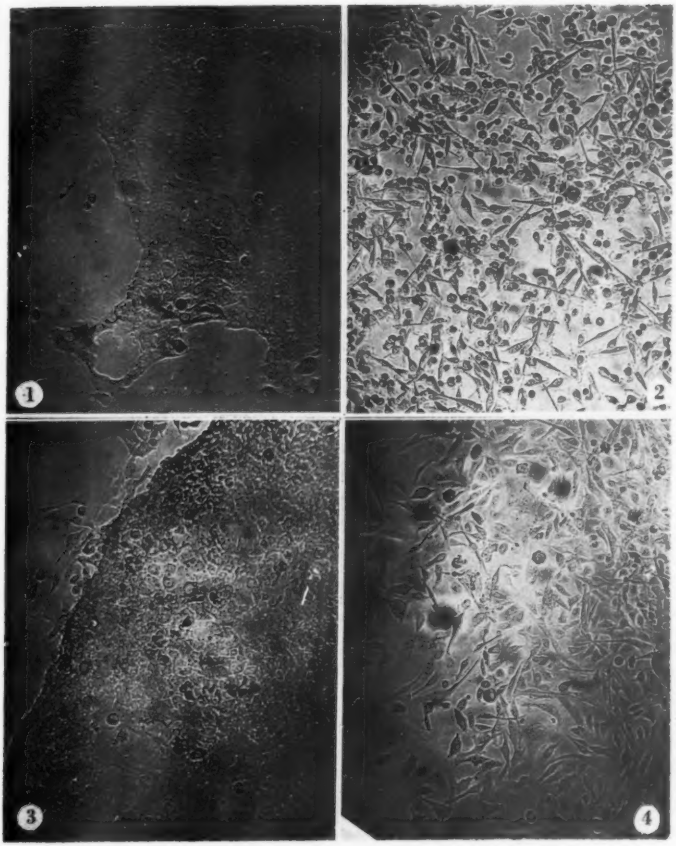


PLATE III

FIG. 1, upper left, shows a membranous portion of a living roller tube colony, growing in dilute human serum, from the HeLa strain of epidermoid carcinoma of the uterine cervix.

FIG. 2 shows cells from the same strain widely dispersed with rounded and partially expanded forms. Cultures grown under similar conditions.

FIG. 3 shows a tissue culture recovery of the HeLa strain from a 3-month guinea pig passage (anterior chamber of the eye) also growing as a membrane under similar conditions.

FIG. 4 shows dispersed cells from the same guinea pig passage HeLa strain grown under similar conditions.

All of these cultures can be readily loosened from the glass by the addition of dilute trypsin and the cell population enumerated directly. All photos of plate III from roller tubes under low power (16 mm.), bright field microscopy. Mag. 120 x.

evidence of a cytopathogenic effect. The cytologist should be reluctant to accept this conclusion. More recently a newly isolated group of viruses associated with acute respiratory illness (Hillman and Werner; Rowe and Associates) and adenoid disease have been shown to be cytopathogenic for the HeLa cell strain. Homotypic antibody has been shown to block the cytopathogenic effects of some of the agents enumerated and permits identification of the virus involved. Further work of this sort with the HeLa and other strains may soon establish the relation of the acute respiratory disease agents to the "common cold" viruses.

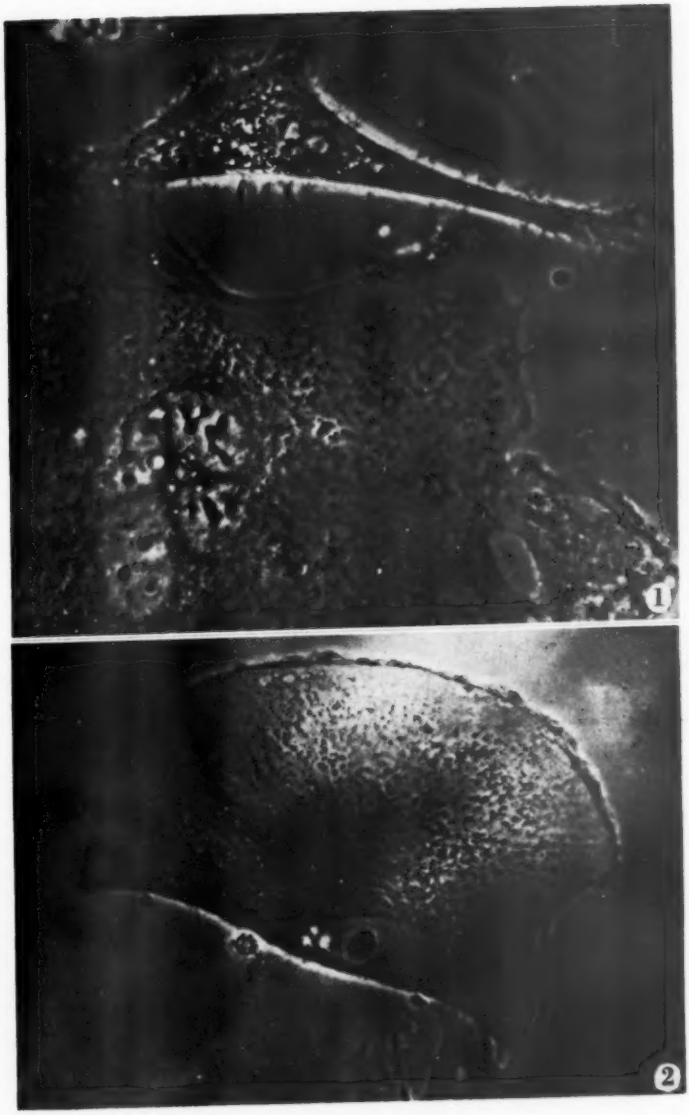
A few examples of apparently futile attempts to grow other viral agents have demonstrated our meager knowledge of virus host-cell relationships. I have studied, with Dr. Bang, a wide variety of normal and malignant cell cultures exposed ideally to infectious (?) material in attempts to isolate a virus from cases of Far Eastern hemorrhagic fever. Even when this was done under field conditions[3] of bedside availability of blood and biopsy material as tissue culture inocula, no evidence of a cytopathogenic effect could be detected in normal and malignant cell strains of humans and rats. An even wider spectrum of cells and media has been explored by Bang, *et al.*, in attempts to isolate the agents of hepatitis. So far, none of these virus agents producing disease in humans has been isolated by tissue culture means.

[3] Field studies in Japan and Korea (Spring, 1953), conducted by Gey and Bang, were sponsored by the Commission on Hemorrhagic Fever of the Armed Forces Epidemiological Board, and supported by the Office of the Surgeon General, Department of the Army. (To be published.)

PLATE IV

FIG. 1 shows two living cells of the HeLa strain of epidermoid carcinoma. The upper contracted cell reveals numerous short sharp microfibrils as the chief surface structures elaborated by the plasma cell membrane. Comparative photomicrographs of living and osmic acid fixed cells show that these structures are comparable to the intercellular bridges. The large cell with four nuclei is thinly spread out and reveals some surface microfolds as another morphologic form of the membrane. Filamentous mitochondria are widely dispersed throughout the cytoplasm. Living image with dark contrast phase microscopy. Mag. 600 x.

FIG. 2 shows a thinly spread out normal epithelial cell of strain LiWi from human adult cervical epithelium. The outer margin is somewhat thicker in a narrow zone and shows some bright inclusion droplets at the upper edge which will soon move toward the juxtanuclear area as they become darker and smaller. Living image *dark contrast* phase microscopy. Mag. 600 x.


1
2

About a year ago, in connection with these hemorrhagic fever studies, we initiated some studies aimed at concentrating viruses on cells under conditions of high gravity centrifugation. This new technique for the deposition of sedimentable materials on living cells under high gravity offers new opportunities for the study of particulate agents where the concentrating of small particulate materials on cells is considered important. The tolerance of several strains of cells to ultracentrifugation is shown in Table 1. It may be seen that the human tumor strains A.Fi.

TABLE 1

Tolerance of Various Strains of Cells to Ultracentrifugation

Exp. No.	Strain	Origin	Cord Serum Conc.	Temp. ‡1.0°C	Time (hrs.)	R.C.F.	Recovery
1	HeLa	H.Ca.	10%	22	0.5	26,500	Very Good
2	A.Fi.	H.Sar.	20%	26–17	3	81,500	Very Good
	14p	Rat Fib.	20%	26–17	3	81,500	Very Good
	72	Rat Sar.	20%	26–17	3	81,500	Poor
3	A.Fi.	H.Sar.	20%	36.5	3	81,500	Very Good
	14p	Rat Fib.	20%	36.5	3	81,500	Very Good
	72	Rat Sar.	20%	36.5	3	81,500	Very Good
4	A.Fi.	H.Sar.	20%	36.5	3	81,500	Very Good
	HeLa	H.Ca.	20%	36.5	3	81,500	Very Good
	14p	Rat Fib.	20%	36.5	3	81,500	Very Good
	72	Rat Sar.	20%	36.5	3	81,500	Very Good
5	A.Fi.	H.Sar.	20%	36.5	3	111,000	Good
	HeLa	H.Ca.	20%	36.5	3	111,000	Good
	14p	Rat Fib.	20%	36.5	3	111,000	Good
	72	Rat Sar.	20%	36.5	3	111,000	Good

The cell strains listed recovered completely in all the experiments. The "Poor" recovery given for strain 72, Experiment 2, refers only to the early response a few days after centrifugation and was a temporary effect.

R.C.F. = Relative Centrifugal Force (gravities).

H.Ca. = Human Cancer.

H.Sar. = Human Sarcoma.

Rat Fib. = Rat Normal Fibroblast.

Rat Sar. = Rat Sarcoma.

and HeLa and the rat strains 14p (normal origin) and 72 (tumorous origin) tolerate high gravity for long periods over a wide range of temperatures. It must be concluded that cultured mammalian cells tolerate hours of exposure at body temperature when subjected to the forces operating at over 100,000x gravity. By this technique we have been able to sediment viruses directly

on living cells in an effort to demonstrate maximum virus destruction as early as possible.

Before closing these comments relating tissue culture to virus growth, a few comments should be made about our limited experiences with the so-called tumor viruses. So far in our experience, virus-cell relationships in tissue culture of the Rous sarcoma or the agent with normal fibroblasts has revealed only a destructive action of the virus on cultured strains of chick fibroblasts grown in either heterologous or homologous media. Others have reported (Sanford and associates, Tenenbaum and Doljanski) on various aspects of this problem but have not stressed the cytopathogenic effects of the virus. There is some giant cell production, but these, like the fibroblasts, rapidly peter out in continuous cultures. In this preliminary work of Dr. Wilson Lo, of our laboratories, an effort is being made to determine the conditions necessary *in vitro* for the active proliferation of these virus infected cells, similar to that found *in vivo*.

Some work has been done also with the Vx-2 carcinoma of the rabbit. This work has been pursued further by Dr. Henri Febvre with the kind cooperation of Dr. Peyton Rous. Both the Vx-2 and the Vx-7 carcinomas are difficult to maintain in continuous culture. The Vx-2 carcinoma cells which grow poorly for long periods show large inclusions in cultures. Their nature and relation to papilloma virus infection have not been revealed.

For the study of strictly comparable autologous normal and malignant cells in continuous culture, there has been a need for a better selection of human tissues which are both accessible and continually available. Normal and malignant cells of epidermal origin are ideal for such studies, but the long-term tissue culture responses have been rather poor. In spite of this, we have succeeded in establishing for varying periods some strains of normal and malignant cells from the uterine cervix. They have at least given us a good start in our studies on the cytology and proliferative capacity of early and late carcinoma of the cervix uteri in comparison with its normal autologous epithelium.

Our oldest carcinoma strain, HeLa, now maintained over 3 years in continuous cultures, grows abundantly in a variety of media substrates, including fluid cultures, with cells grown directly on glass using only diluted (30 to 50 per cent in B.S.S.—Gey) placental cord or adult human serum, horse serum, or

guinea pig serum as fluid medium. Sometimes profound changes occur when these cells are maintained in such heterologous media (horse, guinea pig). As yet, the nature of the changes is not clearly understood. Indeed, in some cases, a "persistent virus" has been thought to account for the changes found during prolonged cultivation. The HeLa strain will also tolerate the violent action of continuous agitation when freely suspended cell cultures are maintained in agitated fluid medium. During such cultivation, many colonies stick to the glass and are unaffected by the turbulent medium. This human malignant strain HeLa has been widely distributed throughout this country and abroad, especially for virus studies and studies of cell nutrition, and special cytology. In the latter category, we discovered, fortuitously, in connection with the Far East studies, the ability of these cells to manifest synchronous mitosis following a period of cooling and subsequent re-incubation. This important finding has led us into an evaluation of cellular variations in radiosensitivity correlated with some control of mitosis in malignant cell populations.

The normal cervical epithelium autologous to that of strain HeLa was grown well over a year as a rapidly keratinizing cell strain, but grew only about 5 per cent as rapidly as its malignant derivative. A post-irradiation, autologous "normal" area yielded a slowly growing cell strain composed of small epithelial cells quite different from the malignant and the normal prototypes, thus indicating that the gamma radiation which had wiped out the malignant cells was not without a permanent effect upon the surviving normal cells. We have repeatedly attempted to isolate a vigorous strain of normal epithelial cells of the cervix from other cases but have succeeded only in isolating slowly growing keratinizing cell lines which are most difficult to maintain.

In addition to the HeLa strain of carcinoma cells, other lines have been isolated and maintained for long periods of time, but none, so far, has approached the HeLa strain in its rapidity of growth. As judged from tissue culture studies alone, of this particular tissue, it would appear that a spectrum of malignant cell types may arise from the cervical epithelium whose cytology and proliferative capacity vary.

Heterologous transplantation of only one of these malignant cell strains (HeLa) in the guinea pig's eye has been demon-

strated so far. A tissue culture recovery from a guinea pig passage of this strain has been isolated also. Its cytology and growth characteristics appear to be unaltered after a four-month sojourn in the heterologous guinea pig soil. Phase, cinephase, and electron microscope studies have revealed the presence of numerous microfibrils (Plate IV, Fig. 1) on the surface of these cells which correspond to the microfibrils (intercellular bridges) found in ultrathin sections of carcinoma of the cervix. These and other cytological aspects are being studied.

The marked growth potentialities of the HeLa strain would appear to be related to the polyploid character of the cells and aggressive feeding properties, and both contribute, apparently, to its autonomous behavior. The irregular polyploidy is not greatly different from that found in many other cell strains that we have studied and to which reference has been made on previous occasions for some of them. Some have claimed, as Painter, that greatly enlarged hyperchromatic nuclei have only one ultimate fate; *i.e.*, autolysis. Our earlier work referred to and the current work on the HeLa strain are not in agreement with such a conclusion, since very excellent motion pictures demonstrate the ability of even the most bizarre polyploid cells to divide and produce unequal sized daughter cells, some of which are mixtures of aneuploids, diploids, or polyploids. The statements of Painter no longer hold on this question of the growth vigor of abnormal hyperchromatic cells.

In many of our evaluations of the structural and functional organization of living cells with phase and electron microscopy, we have found the dynamic approach through the use of cinephase microscopy to be of great value. Continuously cultured normal and malignant cells of the same histogenic origin provide excellent material for comparative cytological studies of living and fixed cells. In thinly spread out areas, portions of living cells show under phase microscopy and with the aid of motion pictures a definitive endocellular traffic, apparently due to the activity of the streaming hyaloplasm. High power observations of morphologically different living cell components of the plasma cell membrane, such as the filamentous fibrils and the thin membranous pseudopodia (ruffles) and the early formation of inclusion vacuoles, allow a good interpretation of comparative

electron microscope images of the same structures when studied in the osmic fixed cell at still higher powers.

Our cinephase observations reveal exceedingly well the ability of cells to produce either innumerable membranous pseudopodia or abundant filamentous fibrils at their thinned out edges and on their broad surfaces. Long before the days of phase microscopy, we produced motion pictures of cells which revealed even with bright field microscopy that some mesenchyme cells were covered with a "furry" coating of very fine microfibrils. Motion pictures taken by phase microscopy constantly available for repeated study demonstrate clearly the ability of cells to ingest droplets of the fluid medium (pinocytosis—Lewis) and particulate matter (phagocytosis). The fluid droplets, originally very irregular, soon get beneath the limiting plasma cell membrane, rapidly round up (Plates V and VI), and within a matter of a few minutes are reduced remarkably in size. We have observed a tenfold decrease in a matter of ten minutes. With this reduction in size there is a gradual change in refractive index leading to a fast reversal of the phase visual display from black to white or vice versa, depending on the phase contrast system used. These droplets now take on the hue possessed by the mitochondria. Particles of this sort become associated with mitochondria and appear to fuse with them. Other particles, as they move toward the nucleus, may tear their way through mitochondria which they may have dragged for a considerable distance. Low power images, followed in motion pictures, demonstrate that these particles are swept by fluid currents to the juxtanuclear area. The streaming hyaloplasm may be responsible for the continuously rotating nuclei seen earlier, in movies first shown in 1939 by our laboratory, in rat tumor cells. Pomerat has recently reported on the presence of rotating nuclei in human normal nasal epithelium. Our movies also show rotating nuclei in the HeLa strain.

Returning to the surface activity and structures found there, it is quite possible to see a definite relationship between the microfibrils extending from the surface and the resolvable living fibrous strands deeper in the cell (Plate VII, Figs. 1 and 2). The filamentous fibril base is continuous with the plasma cell membrane at times, or may extend into the living fibrous strands. Oftentimes these strands are so well delineated as parallel bands

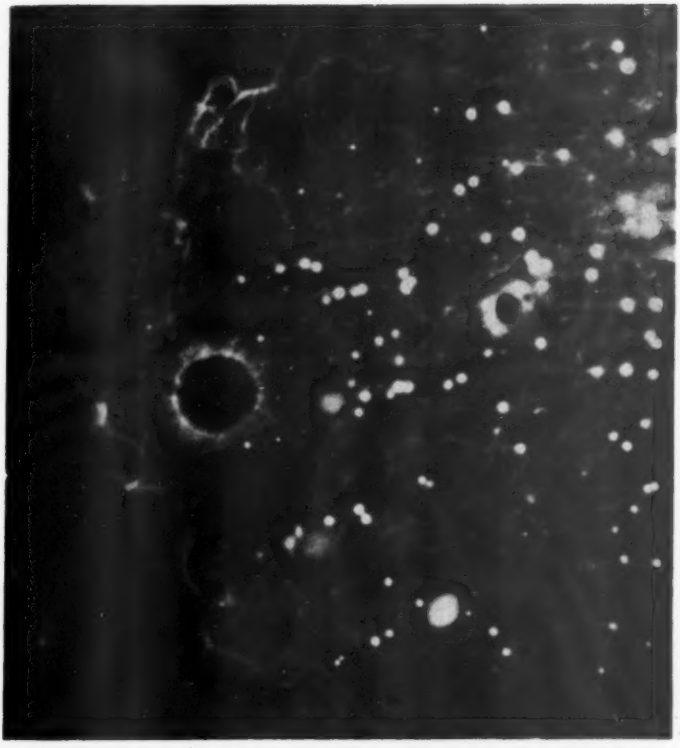


PLATE V

Plate V shows a small portion of a cell of the human chondromyxosarcoma strain D-1 Re taken with *bright contrast* phase microscopy under oil immersion in the living state. A dark rounded inclusion droplet, surrounded by a halo of dispersed light, has just entered the cytoplasm as a result of the trapping processes (pinocytosis—Lewis). Within a matter of ten minutes this mass will move half-way across this field and will be reduced in size with a change in refractive index and a reversal of the phase visual display. At this later period, it would have the same size and appearance as the bright mass seen in the right field of the photograph. Both surface folds and mitochondria make up the linear display. The numerous smaller bright masses scattered about originated in the same manner from previously ingested darker fluid masses. Such inclusion droplets, propelled by protoplasmic streaming, distort and even tear mitochondria as they move toward the juxtanuclear zone. Mag. 3000 x.

that it is possible to see hyaloplasmic streaming in opposite directions in adjacent, well-delineated areas. The individual beads of the beaded mitochondria of some cells are indistinguishable

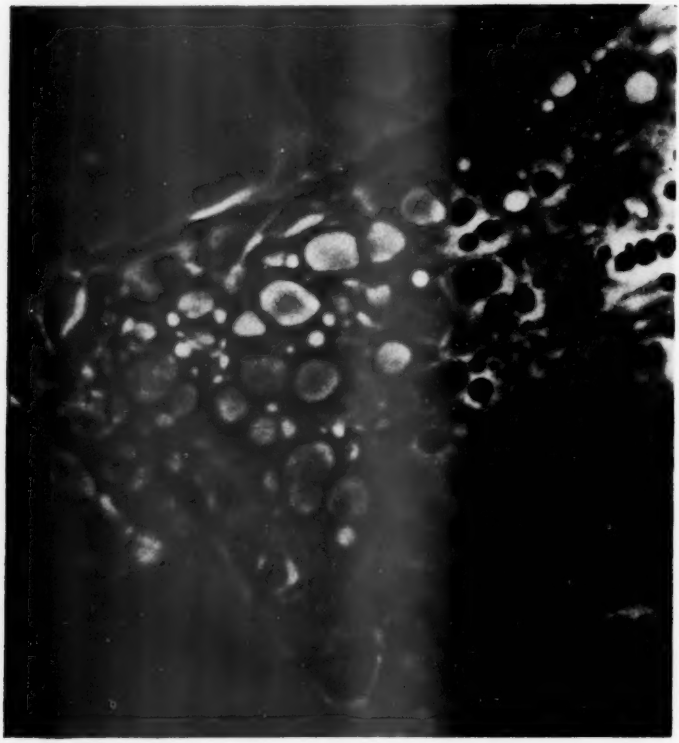


PLATE VI

Plate VI shows a somewhat thickened portion of a cell from the HeLa strain of epidermoid carcinoma of the cervix. This thick region shows many inclusion droplets entering the cytoplasm. These will show rapid change in size and reversal of phase visual display as they move inward and affect the shape and length of mitochondria as previously noted. Oil immersion preparation with *dark contrast* phase microscopy in the living state. Mag. 3000 x.

from the condensed inclusion droplets formed by the action of the hyaloplasm on them earlier and are quite indistinguishable from each other when the droplets are reduced to comparable size. The stream flow appears to sweep all these particles (*i.e.*,

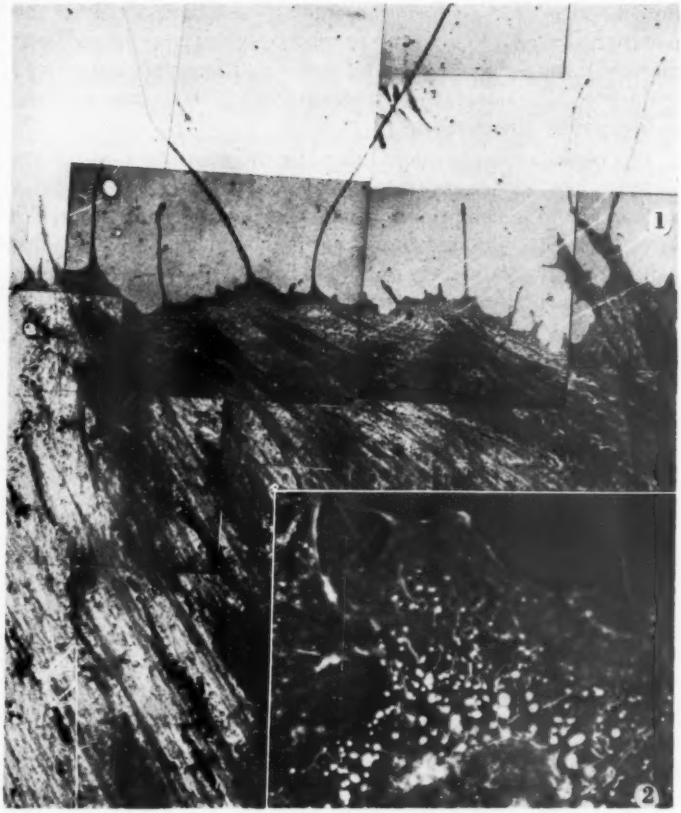


PLATE VII

FIG. 1 shows the margin of a rat normal fibroblast, strain 14p, showing at its thinned out edge long, fiiamentous microfibrils, some of which have a broad base, and in a few instances are continuous with the array of fibrillar structures so well displayed throughout the thinned out cytoplasm. Most of the mitochondria in this region of the cell appear to align themselves with the more or less parallel fibrous bands. Very few inclusion droplets are seen in this peripheral margin. This cell was grown directly on the cover glass in 30 per cent cord serum. Osmic acid vapors fixation for 10 minutes. Mag. 6000 x.

FIG. 2 insert is approximately one-half of a thinly spread out cell of the same strain showing a free border with several living microfibrils which have grown from the free edge in a few minutes or even less. In general, they may be found waving about and often fuse with the plasma cell membrane, especially when observed on the surface. This living cell shows no regions of parallel stress to delineate the mitochondria as seen in some living cells. Nucleus is in lower center. Living image, *bright contrast* phase microscopy. Mag. 1500 x.

mitochondria and inclusion droplets) back and forth in the partitioned areas. Very local, rapid movement may be actuated directly through local molecular forces and therefore not related to the over-all hyaloplasmic streaming which can be best assessed at low power observation.

The organization of the living cell components seen in motion pictures can be demonstrated to match very well comparable regions found in cells viewed under the electron microscope. We have actually compared the same regions of one and the same cell (strain 14p) and have found the living oil-immersion images to match structure for structure those resolved in osmic fixed preparations and imaged with the electron microscope. Many distortions in the mitochondria, such as lateral budding, swellings, and thinned-out filaments, can easily be accounted for by the profound changes produced by forces external to the mitochondria. Other deformations or changes in shape, shown by Fredéric and Chèvremont, may be related to local osmotic pressure changes. The dominant changes that we see in mitochondrial morphology are apparently related to the effects of the local streaming of the hyaloplasm acting directly or indirectly through inclusion droplet movement. For more exacting interpretations, one must resort to a comparison of oil immersion, phase microscopy, electron microscopy, and thin-sectioning on those parts of the cell whose structure and behavior are under scrutiny. Plate VII shows at relatively low power some aspects of the comparable images of cell structure seen in the normal rat fibroblast strain 14p. Our comparative studies with phase and electron microscopy of living and fixed cells have, for several years, provided us with a most valuable set of tools and procedures for a better analysis of the cell's behavior and ultrastructure.

Summary

The use and management of normal and malignant cell strains as source material for a variety of studies are described with reference to (1) the broad spectrum of cells that can now be maintained in continuous cultures. These include (a) a variety of embryonic and extra-embryonic tissues, (b) a variety of adult tissues, and (c) a variety of tumor tissues. Our ability to main-

tain stable strains of cells from many of these tissues is demonstrated here chiefly through the use of human cell strains. (2) The growth responses in simple biologic media, such as dilute serum, which can maintain many strains of cells in continuous culture. (3) An evaluation of some aspects of the structural organization of cells as revealed by comparative studies with phase and electron microscopy. (4) Some determinations of the spectrum of viral susceptibility and their use in studies on virus growth, isolation, and identification, including some results of a new method for evaluating the tolerance of various strains of cells to ultracentrifugation. By this method, viruses can be sedimented directly on living cells at various temperatures. (5) Some results on the isolation of human strains of normal and malignant epithelium as part of a study on an evaluation of the proliferative capacity and cytology of cells from specific tissues, such as the epithelium of the uterine cervix.

The importance of a dynamic approach through cell and virus cultivation toward the solution of problems in cellular physiology and pathology becomes ever more manifest as new cytological disciplines are brought to bear upon them.

Acknowledgment

We are deeply indebted to Mr. Peter Sapranauskas for his help in the production of the excellent phase photographs, some of which (Plates V, VI, and VII) were enlargements made from his very fine motion picture records, and for his help on the ultracentrifugation. The composite electron micrograph was kindly prepared by Mr. Emil Borysko.

The examples of growth of germinal epithelium and strain HeLa cells were made from cultures maintained by Dr. Maria Svotelis and originally explanted by Mrs. Mary T. Kubicek. Without their devoted efforts, these results would not have been attained. We are grateful for their help.

REFERENCES

Bang, F. B., and G. O. Gey: A Fibrillar Structure in Rat Fibroblasts as Seen by Electron Microscopy. *Proc. Soc. Exp. Biol. Med.*, 69: 86–89, 1948.

Bang, F. B., and G. O. Gey: Viruses and Cells, A Study in Tissue Culture Applications. II. Effect of Several Viruses on Cell Types and the Amount of Virus Produced. *Trans. N. Y. Acad. Sci.*, 13: 324–327, 1951.

Bang, F. B., and G. O. Gey: Comparative Susceptibility of Cultured Cell Strains to the Virus of Eastern Equine Encephalomyelitis. *Johns Hopkins Hosp. Bull.*, 91: 427–461, 1952.

Chévremont, M., and J. Frédéric: Évolution des Chondriosomes lors de la Mitose Somatique. Etudiée dans des Cellules Vivantes Cultivées in Vitro par Microscopie et Microcinématographie én Contraste de Phase. (2e partie). *Archives de Biologie*, 63: 259–277, 1952.

de Ruyck, R.: Transmission du Virus Choriotrope au Poulet Adulte. Production de Reticuloses et Reticulo-endothelioses Malignes. *Bulletin du Cancer*, 49: 78–86, 1953.

Fell, H. B., and E. Mellanby: Metaplasia Produced in Cultures of Chick Ectoderm by High Vitamin A. *J. Physiol.*, 119: 470–488, 1953.

Frédéric, J., and M. Chévremont: Recherches sur les Chondriosomes de Cellules Vivantes par la Microscopie et la Microcinématographie en Contraste de Phase. (Ire partie). *Archives de Biologie*, 63: 109–131, 1952.

Furth, J., Conditioned and Autonomous Neoplasms: A Review. *Cancer Res.*, 13: 477–492, 1953.

Gaillard, P. J.: Growth, Differentiation, and Function of Explants of Some Endocrine Glands. *Symposia Soc. Exper. Biol.*, Cambridge Univ. Press 2: 139–144, 1948.

Gey, G. O.: The Maintenance of Pure Strains of Human and Animal Tumor Cells in Culture. *Int. Cancer Res. Fdn.*, (3d Sci. Report, p. 34), 1935.

Gey, G. O., and M. K. Gey: The Maintenance of Human Normal Cells and Human Tumor Cells in Continuous Culture. I. Preliminary Report: Cultivation of Mesoblastic Tumors and Normal Tissue and Notes on Methods of Cultivation. *Am. J. Can.*, 27:45–76, 1936.

Gey, G. O., M. K. Gey, F. Inui, and H. Vedder: Effects of Crude and Purified Penicillin on Continuous Cultures of Normal and Malignant Cells. *Johns Hopkins Hosp. Bull.*, 77: 116–131, 1945.

Gey, G. O., J. H. Hanks, and R. Barrett: Retardation of Growth and Metabolism of Normal and Malignant Cells During Continuous Cultivation. *Growth*, 12: 69–105, 1948.

Gey, G. O., M. K. Gey, W. M. Firor, and W. O. Self: Cultural and Cytologic Studies on Autologous Normal and Malignant Cells of Specific *in vitro* Origin. Conversion of Normal into Malignant Cells. Internat. Union Cancer, Paris, Acta 6: 706–711, 1949.

Gey, G. O., and F. B. Bang: Cell Structure. A Comparative Study of the Cytological Characteristics of Normal and Malignant Cells With Phase and Electron Microscopy. *Anat. Rec.*, 109: 411, 1951.

Gey, and F. B. Bang: Viruses and Cells. A Study in Tissue Culture Applications. I. Cells Involved—Availability and Susceptibility. *Trans. N. Y. Acad. Sci.*, 14: 15–24, 1951.

Gey, G. O., W. D. Coffman, and M. T. Kubicek: Tissue Culture Studies of the Proliferative Capacity of Cervical Carcinoma and Normal Epithelium. *Cancer Res.*, 12: 264–265, 1952.

Gey, G. O., F. B. Bang, and M. K. Gey: Responses of a Variety of Normal and Malignant Cells to Continuous Cultivation and Some Practical Applications to the Biology of Disease. *Trans. N. Y. Acad. Sci.*, 1954 (in press).

Hilleman, M. R., and J. H. Werner: Recovery of New Agent from Patients With Acute Respiratory Illness. *Proc. Soc. Exper. Biol. and Med.*, 85: 183–185, 1954.

Lewis, W. H.: Pinocytosis. *Johns Hopkins Hosp. Bull.*, 49: 17–27, 1931.

Morgan, J. F., H. J. Morton, and R. C. Parker: Nutrition of Animal Cells in Tissue Culture. I. Initial Studies on a Synthetic Medium. *Proc. Soc. Exper. Biol. and Med.*, 73: 1, 1950.

Novick, A., and L. Szilard: Description of the Chemostat. *Science*, 112: 715, 1950.

Owens, Olga v. H., G. O. Gey, and M. K. Gey: A New Method for the Cultivation of Mammalian Cells Suspended in Agitated Fluid Medium. *Proc. Am. Assoc. Cancer Res.*, 1. 41, 1953.

Owens, Olga, M. K. Gey, and G. O. Gey: Growth of Cells in Agitated Fluid Medium. *Trans. N. Y. Acad. Sci.*, 1954 (in press).

Painter, T. S.: Some Cytological Aspects of the Nucleic Acid Problem. *Tex. Rep. on Biol. and Med.*, 11: 641–778, 1953.

Pomerat, C. M.: Rotating Nuclei in Tissue Cultures of Adult Human Nasal Mucosa. *Exper. Cell Res.*, 5: 191–196, 1953.

Pogogeff, I. A., and M. R. Murray: Form and Behavior of Adult Mammalian Skeletal Muscle *In Vitro*. *Anat. Rec.*, 95: 321–335, 1946.

Rosenberg, S., and P. L. Kirk: An Ultrafiltrate Growth Factor for Tissue Culture. *Science* 117: 566, 1953.

Rosenberg, and P. L. Kirk: Tissue Culture Studies. Identification of Components and Synthetic Replacements for the Active Fraction of Chick Embryo Extract Ultrafiltrate. *J. Gen. Physiol.*, 37: 239–248, 1953.

Rowe, W. P., R. J. Huebner, L. K. Gilmore, R. H. Parrott, and T. G. Ward: Isolation of a Cytopathogenic Agent from Human Adenoids Undergoing Spontaneous Degeneration in Tissue Culture. *Proc. Soc. Exper. Biol. and Med.*, 84: 570–573, 1953.

Sanford, K. K., G. D. Likely, W. R. Bryan, and W. R. Earle: The Infection of Cells in Tissue Culture With Rous Sarcoma Virus. *J. Nat. Cancer. Inst.*, 12: 1317–1343, 1952.

Scherer, W. F., J. T. Syverton, and G. O. Gey: Studies on the Propagation *In Vitro* of Poliomyelitis Viruses. IV. Viral Multiplication in a Stable Strain of Human Malignant Epithelial Cells (Strain HeLa) Derived from an Epidermoid Carcinoma of the Cervix. *J. Exper. Med.*, 97: 695–710, 1953.

Scherer, W. F., and J. T. Syverton: Cytologic Effects *In Vitro* of Epitheliotrophic and Encephalitogenic Viruses on Human Epithelial Cells, Strain HeLa. *Fed. Proc.*, 13: 511, 1954.

Stoler, M., and M. K. Gey: Destruction of a Human Fibrosarcoma Strain (A.Fi.) in Tissue Cultures Following the Growth of Poliomyelitis Viruses. *Johns Hopkins Hosp. Bull.*, 92: 385–393, 1953.

Tenenbaum, E., and L. Doljanski: Studies on Rous Sarcoma Cells Cultivated *In Vitro*. II. Morphologic Properties of Rous Sarcoma Cells. *Cancer Res.*, 3: 585–603, 1943.

White, P. R.: Cultivation of Animal Tissues *In Vitro* in Nutrients of Precisely Known Constitution. *Growth*, 10: 231, 1946.

EVIDENCE OF THE FUNCTIONAL DIFFERENTIATION OF THE EMBRYONIC CHICK THYROID IN TISSUE CULTURE*

FEDERICO GONZALES

Excellent reviews of the literature on differentiation in tissue culture are available (*e.g.*, Fell, 1951). Therefore, I shall mention here only those papers which bear directly on my problem.

In regard to the functional differentiation of the chick thyroid *in vivo*: Trunnell and Wade of this institution have shown by the use of radioactive iodine and filter paper chromatography that the gland first starts to produce diiodotyrosine and thyroxine about the end of the 9th day of incubation.

Dr. Esther Carpenter of Smith College reported in 1942 some studies on the differentiation of chick thyroid in tissue culture. She explanted glands from 8-day embryos and found by a study of fixed and strained section of the cultures that the morphological differentiation of the explanted thyroid parallels closely its development *in vivo*.

In attempts to determine whether the cultures were producing an active hormone, she fed cultures to tadpoles and observed the tadpoles for an effect on metamorphosis. She reported that the results of feeding experiments were inconclusive. She gave no further details.

Other workers have attempted to demonstrate thyroid activity in tissue culture, but they used already differentiated glands. Thus, Ebeling (1924) grew thyroid epithelium and fibroblasts in confronting cultures, that is, he cultivated explants of thyroid epithelium and fibroblast explants within the same clot. He found that fibroblast cultures grown in the presence of thyroid tissue showed an increase in growth over those grown in the absence of thyroid tissue.

It was judged worth while to make some studies on the factors affecting the functional differentiation of the chick embryo

* From The University of Texas, M. D. Anderson Hospital and Tumor Institute, Houston. This work was supported in part by funds from the American Cancer Society (Instr. 23-F). Presented at the Eighth Annual Cancer Symposium, April, 1954, at the M. D. Anderson Hospital, Houston.

thyroid in tissue culture. The results which I will present here are the outcome of preliminary experiments along these lines. The following experiment was made in an attempt to answer the first question that arises in a study of this kind, namely, does the embryonic chick thyroid have the capacity to differentiate *functionally* in tissue culture?

Procedure. Thyroids were taken from 7-day embryos and collected in a Petri dish containing sterile tyrode solution. Any excess connective tissue was trimmed away with fine knives.

The glands were then explanted, one per clot, and cultivated by the Maximow double coverslip method. The original clot was composed of 25 per cent Embryo Juice (from 11-day embryos) 25 per cent Tyrode and 50 per cent Plasma. The cultures were washed and fed every 2 days.

Twenty-four hours before being harvested, the cultures received one drop each of a solution of radioactive iodine in Tyrode. The activity of radioactive iodine in the saline was 100 μ curies/ml. Assuming that there were 20 drops of equal size per ml., a rough estimate of the dose received by each culture would be 5 μ curies of I^{131}.

Following the iodine treatment, the cultures were scraped into small centrifuge tubes containing acetone. Four cultures were pooled in each tube and left in the acetone over night. The precipitate was then washed with acetone and then with water. Two molar NaOH was added to the tubes which were then placed in a hot water bath. The hydrolysis was allowed to proceed for 16 hours. Following this, the pH of the hydrolysate was adjusted to a value of 3 by the dropwise addition of 6M H_2SO_4. The acidified hydrolysate was extracted with butyl alcohol.

Next, following the method of chromatography developed by Taurog, Tong, and Chaikoff (1950), the butanol extract was plated on a line a short distance from one end of a strip of filter paper. The end of the paper bearing the dried extract was dipped into a collidine-water mixture in a chromatography jar. A small beaker of concentrated ammonium hydroxide was included in the jar to provide an atmosphere of ammonia. The papers were left in the sealed chromatography jars for 16 hours after which they were air dried.

In order to localize the radioactive iodinated compounds, the dried chromatograms were put opposite X-ray film in film

holders and allowed to expose in the dark for 2 weeks. At the end of the exposure period, the film was developed and the positions of darkened areas compared with those of known compounds as determined by color test.

Results and Discussion

Figure I shows chromatograms of hydrolysates of cultures of 7-day embryo thyroids which have been cultured for 1 day through 5 days. The symbols on the left refer to the various compounds.

We see here that the first chromatogram (1 day) shows the presence of monoiodotyrosine (MIT) and inorganic iodide (I) only, whereas the second (2 days) shows the presence of diiodotyrosine (DIT) and thyroxine (Tx) in addition to the MIT. This would indicate that the explanted thyroids begin to produce DIT and Tx at some time between the end of the first day and the end of the second day in culture.

Up to this point the behavior of the thyroid *in vitro* closely parallels its behavior *in vivo*. You will recall that Trunnell and Wade found that the gland *in vivo* begins to produce these compounds at about 9 days incubation. Thus, according to these preliminary results the explanted thyroids begin to produce DIT and Tx at the same time as they would had they remained in the embryo.

But after this the story changes. A comparison of the 5 chromatograms shown in figure I indicates that the production of DIT and Tx decreases after having reached a maximum in the second day.

This decrease in activity could be due to several factors. There are indications that one of the factors is insufficient thyrotropic hormone.

Rawson stated in a personal communication that in 1939–40, he cultivated thyroid from 18- and 19-day chick embryos. With the aid of I^{131} he found that thyroid explants which were grown in the absence of thyrotropic hormone showed weak iodine concentrating ability compared with those grown in a medium containing ¼ unit of thyrotropic hormone per cc. of medium.

Other evidence which suggests a too low thyrotropic hormone level as a factor in this decrease in activity is the work of Fugo

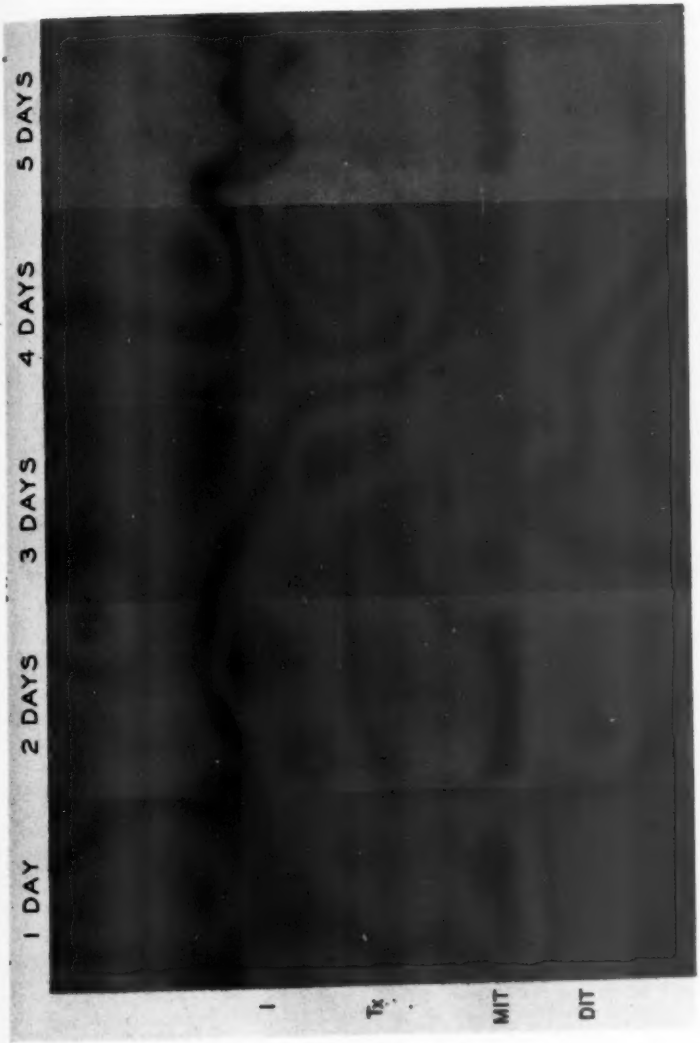


FIG. 1. Chromatograms of hydrolysates of thyroid explants after 1 through 5 days of cultivation. See text for explanation.

(1940) on the effects of hypophysectomy on the development of the chick. He found that the thyroids of hypophysectomized embryos develop histologically up to a point and then regress.

Summary. Preliminary experiments indicate that the thyroids from 7-day chick embryos behave in tissue culture essentially as they do *in vivo* for the first 2 days, as judged by their production of diiodotyrosine and thyroxine. After the second day production of these compounds stops or is greatly reduced.

Acknowledgments

The author wishes to thank Dr. Jack Trunnell and Mr. Paul Wade for their valuable help and suggestions.

REFERENCES

Carpenter, Esther: Differentiation of Chick Embryo Thyroids in Tissue Culture. *Jour. Exp. Zool.*, 89: 407–431, 1942.

Ebeling, A. H.: Action de l'epithelium thyroiden en culture pure sur la croissance des fibroblasts. *Soc. Biol. Compt. rend.*, 90: 1449–1450, 1924.

Fell, Honor B.: *Histogenesis in Tissue Culture*, in *Cytology and Cell Physiology*, 2nd ed. (G. H. Bourne, Editor), Oxford University Press, London, 1951.

Fugo, Nicholas W.: Effects of Hypophysectomy in the Chick Embryo. *J. Exp. Zool.*, 85: 271–297, 1940.

Rawson, R. W.: Personal communication.

Taurog, A., W. Tong, and I. L. Chaikoff: The Monoiodotyrosine Content of the Thyroid Gland. *J. Biol. Chem.*, 184: 83–97, 1950.

Trunnell, J. B., and Paul Wade: Factors Governing the Development of the Chick Embryo Thyroid. II. Chronology of the Synthesis of Iodinated Compounds Studied by Chromatographic Analysis (in press). *J. Clin. Endocrinology and Metabolism.*

CYTOLOGICAL STUDIES ON HeLa, A STRAIN OF HUMAN CERVICAL CARCINOMA

I. Observations on Mitosis and Chromosomes*

T. C. Hsu

It is of paramount importance for research workers to have a continuous supply of very closely similar or of identical material so that detailed information can be obtained and repeated experiments can be made with readily comparable results. In experimental animals many tumors can be maintained successfully by repeated transplantation to susceptible young animals. For human neoplasms, however, other methods must be developed. Growing human tumors in rats or mice by the methods of Toolan (1952, 1953) seems to be very promising, but it has not been developed to the point that permits large scale investigations. An alternative way is to experiment with cells *in vitro*. In a previous paper (Hsu, 1954) it was pointed out that cytological studies on human cancers can be made to advantage by culturing surgical specimens and using the hypotonic solution prefixation treatment. However, without a constant supply of certain cell strains the advantage is somewhat limited for continuous experimentation.

Many tissue culture laboratories possess the strain HeLa. This is from a cervical carcinoma and was originally established by Gey and his associates at Johns Hopkins University. With this material widely available it seemed highly desirable to study its various cytological aspects in detail. The present report deals with some preliminary observations on mitosis and the chromosomes of this strain. Dr. Gey kindly provided us with the material for our initial cultures.

Materials and Methods. The HeLa strain was derived from a specimen of an epidermoid carcinoma of the cervix more than

* From the Tissue Culture Laboratory, The University of Texas, Medical Branch, Galveston. Presented at the Eighth Annual Symposium on Fundamental Cancer Research, April, 1954, at the M. D. Anderson Hospital for Cancer Research, Houston. Supported in part by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council administered by C. M. Pomerat and in part by a grant-in-aid from the Damon Runyon Memorial Fund for Cancer Research, Inc., administered by the author.

three years ago. Its history was briefly described by Scherer, Syverton, and Gey (1953). We received a stock labeled HeLa 184 which was a line cultivated in one of Gey's culturing media. This strain has been kept in roller tubes in our laboratory since the summer of 1953 by the following procedure: Subcultures were made every other week by cutting the old cultures into small pieces as explants for the next generation. These were placed on the wall of the roller tubes with a sterile pipette and without a plasma clot. After about two hours, when the explants were dry enough to adhere to the tube wall but when the cells were still alive, 2 ml. of a fluid phase medium was added to each tube and incubation was started. The fluid phase medium was made of 50 per cent human ascitic fluid, 45 per cent Gey's balanced salt solution, and 5 per cent embryonic extract. The culture tubes received changes of fluid medium twice a week.

For the present studies subcultures were imbedded in plasma-embryonic extract clots on 12 x 50 mm. cover slips. Three explants were usually mounted on each cover slip and two such preparations were placed back to back in a roller tube. The fluid phase medium, constituted as described above, was added approximately one hour later. Two sets of cultures were employed for the data of the present article. Subculture 17 (S-17) was maintained in tubes for five days without changing the fluid phase medium and was treated with a hypotonic solution for one hour. The method used was similar to that described by Hsu and Pomerat (1953) except that the hypotonic solution was prepared by mixing one part of Gey's balanced salt solution with nine parts of Gey's solution without NaCl and that the treatment was extended to 60 minutes. Subculture 20 (S-20) was set up in the same way as S-17 but samples were taken every other day up to the 24th day. The fluid phase medium was changed after samples of the eighth and the 14th days had been taken. The cultures were washed in regular Gey's solution, fixed in Navashin's fluid, and stained with acetocarmine for 20 minutes. During the dehydration process, they were counterstained by saturated light green in 96 per cent alcohol for less than one minute. The preparations thus made showed deep red chromosomes and greenish spindles.

Phase contrast, time-lapse cinematography was employed from time to time at various magnifications and a few of the sequences will be mentioned briefly.

Mitosis and Endomitosis. Mitotic figures were very abundant in the early days of incubation (Fig. 1) and even after more than three weeks were still encountered frequently. Metaphase as a rule dominated the populations of the dividing cells. A number of metaphases and anaphases showed a hyaline area around the chromosome plates and the spindles (Fig. 2).

Abnormal mitoses were extremely common, particularly as to multipolar spindles (Fig. 3). However, comparatively few metaphases showed chromosomes arranged off the spindle, hence few anaphases showed lagging chromosomes. In the earlier days of culture many cells exhibited a disturbance of cell division, with chromosomes widely scattered over the spindle apparatus (Fig. 4). Micronuclei were not common and it is assumed that most of these cells either underwent endomitosis and that a single nucleus of a higher order of ploidy was formed in each case or that they eventually divided.

The problem of endomitosis is very complicated. In tissue culture preparations of chick embryonic cells Stilwell (1952) observed endomitotic cells in the outgrowth. She seems to imply that these have an "intact nuclear membrane" even though the endomitosis is in the later stages. Levan and Hauschka (1953) found such cells occasionally in some mouse ascites tumors. In HeLa, such cells were not common. A number of dividing cells in HeLa showed a membrane-like structure around the chromosomes and the spindles, but it was definitely not a nuclear membrane. Figure 5 shows such a membrane-like structure in metaphase, figure 6 in late anaphase and figure 7 shows such a "membrane" even after the cleavage. Occasionally prophases also could be observed to have such a "membrane" outside and in addition to the true nuclear membrane (Fig. 8). In our material nearly all the mitotic cells reaching metaphase possessed a spindle apparatus and no nuclear membrane was observed. From various stages of the spindle and the chromosome development it seems that the endomitotic process in HeLa started from the metaphase. The spindle began to degenerate and the chromosomes, originally arranged in the metaphase plate or plates, began to crowd or even to stick to each other. Later, as the spindle apparatus was

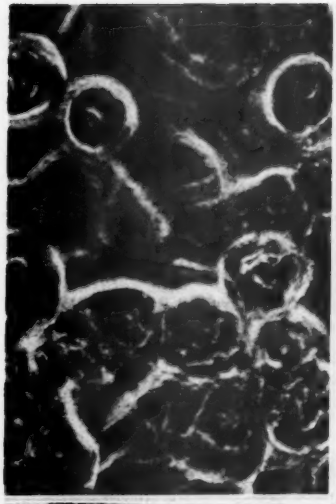


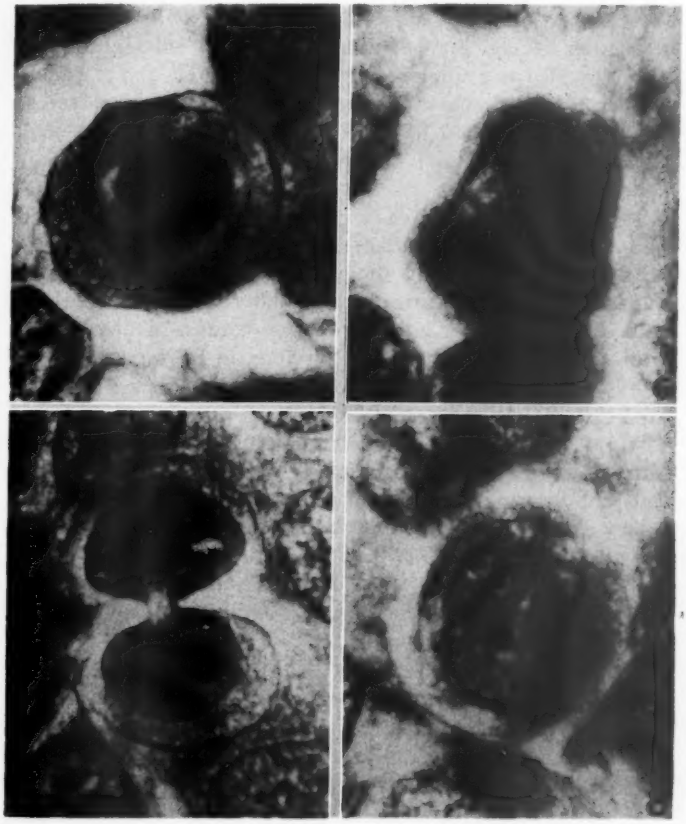
FIG. 1. Phase contrast photograph of a portion of middle area in the outgrowth, showing many cells in mitosis. Four-day-old culture.

FIG. 2. Metaphase, showing the hyaline area around the metaphase plate. Four-day-old culture. Carmine and light green.

FIG. 3. A tripolar metaphase. Four-day-old culture. Carmine and light green.

FIG. 4. An anomalous spindle with scattered chromosome elements. Two-day-old culture. Carmine and light green.

further atrophied the chromosomes lost their identity when they started to despiralize. The situation was somewhat similar to the process described in epidermal cells in tissue culture (Hsu, 1952).



FIGS. 5–8. Cells in mitosis, showing the membrane-like structure. Eight-day-old cultures. Carmine and light green.

FIG. 5. Metaphase.
FIG. 6. Late anaphase.
FIG. 7. Cytokinesis.
FIG. 8. Prophase.

It should be emphasized that the foregoing interpretation on HeLa endomitosis is derived from fixed and stained preparations

only. Our cinematographic records on HeLa mitosis have demonstrated that such a conclusion perhaps cannot be completely relied upon because unexpected changes frequently occur. For instance, in one sequence where the metaphase chromosomes began to crowd together and to stick to each other as if the cell was going to undergo endomitosis, the cell eventually divided. In another sequence an apparently "normal" metaphase suddenly reverted to a single nucleus with no indication of spindle degeneration or changes in the morphology of the chromosomes. Even perfect anaphasic or telophasic chromosome groups would move back to the center of the cell and reunite into a single nucleus.

Observations on more cases of mitosis and endomitosis with the motion picture method are in progress and will be reported in detail at a later date. At the present time it is only fair to say that to draw conclusions from static materials such as fixed and stained preparations is not entirely safe because of the dynamic nature of the living material. Therefore, in the analyses to follow the data only represent what could be determined from the fixed and the stained preparations.

Mitotic Activity. The rate of the mitotic division for the HeLa cultures was estimated by recording the cells in mitotic stages (including possible endomitoses) out of the total cells counted. Each culture was read in three zones, the periphery of the outgrowth, the middle area of the outgrowth, and the area near the explant. Samples were pooled from as many cultures as possible and each sample consisted of over 2000 cells. The results are presented in table 1.

It is evident from table 1 that the rate of mitosis is amazingly high in the early days of incubation, being close to 12 per cent in both the periphery and the middle area of the outgrowth. Even in the area near the explant, which showed a considerably lower rate of mitosis than the outskirts of the culture, the division rate was in the vicinity of 5 per cent. However, the high mitotic indices dropped rapidly after a few days' residence in the unchanged medium, especially in the periphery. The area near the explant had the greatest mitotic activity of all zones after the sixth day, regardless of changes of the fluid phase. The effect on mitosis of changing the fluid phase medium is indicated by an increase of mitotic cells on the tenth and the 16th days. This

occurred after the changes had been made when the eighth and the 14th day samples were taken. Mitotic activity in HeLa persisted for a comparatively long period, at least up to the middle of the fourth week, and there is no reason to suspect that that time was the end of cellular proliferation.

TABLE 1

Mitotic indices of HeLa cultures. Each sample consists of over 2,000 cells. Arrows indicate time of changing fluid phase medium.

Days of culture	Periphery	Middle area	Area near explant
2	11.91	11.23	5.22
4	4.68	3.51	4.81
6	1.87	2.44	3.30
8	0.93	1.61	1.94
→			
10	0.82	2.89	3.98
12	0.84	2.49	2.87
14	0.96	1.81	1.77
→			
16	1.07	2.34	2.90
18	0.69	1.89	2.37
20	0.88	1.87	2.05
22	0.27	1.90	2.31
24	0.52	1.37	1.74

The Prophase Coefficient. Timonen and Therman (1950) compared the relative frequencies of prophase, metaphase and anaphase in epithelial cells of both malignant and nonmalignant tissues of the genital tract of women and made the important discovery that the prophase of malignant tissues is, as a rule, represented by fewer cells than that of the nonmalignant ones. In a given cell population, if there is nothing to influence the duration of a particular stage, such as the arrest of metaphase by colchicine, it can be assumed reasonably that the relative proportions of different stages closely represent their relative duration. Therefore when prophase numbers are fewer in cancer tissues it would mean that neoplastic cells have a shorter prophase. In table 2 which is a day-by-day summary of the proportion of different mitotic stages in HeLa S-20, the proportion of prophases seems also to be low. Moreover, the percentage of prophase increased when the incubation period was over one week.

For a better presentation of this phenomenon we propose to compare the prophase-metaphase duration by an arbitrary figure, the prophase coefficient, which is calculated by dividing the number of cells in prophase by those in metaphase. If the duration of the two stages is about equal, the coefficient should approach unity, and the smaller the coefficient the shorter the prophase or the longer the metaphase. The two columns of prophase and metaphase in table 2 are recalculated as coefficients and are presented in table 3 with significance tests for each

TABLE 2

Percentage of division stages in HeLa cultures. Arrows indicate time of changing fluid phase medium.

Days of culture	Prophase	Metaphase	Anaphase	Telophase	Restitution daughter nuclei	Endomitosis	Cells examined
2	13.32	51.46	9.31	6.20	11.68	8.03	548
4	13.86	44.19	9.55	5.99	13.86	12.55	534
6	13.95	46.77	7.99	3.91	14.29	13.09	588
8	22.89	37.61	5.39	3.79	18.22	12.10	686
→							
10	23.80	37.77	6.52	4.52	15.29	12.10	752
12	16.81	37.93	6.03	4.31	20.55	14.37	696
14	19.25	43.25	5.75	3.25	18.00	10.50	400
→							
16	20.84	37.48	6.69	3.63	15.49	15.87	523
18	20.04	41.16	5.17	3.45	14.87	15.30	464
20	22.27	43.72	7.89	3.44	12.55	10.12	494
22	21.05	47.37	5.99	1.45	11.62	12.52	551
24	20.63	43.42	5.30	3.14	15.91	11.59	509

two-day period calculated by χ^2. With one degree of freedom the χ^2 value between the sixth day and the eighth day is highly significant. In the stained preparations it was also noted that late prophases were very frequently observed only after the eighth day of culture. The prophasic figures prior to this were mainly in the early stages, with occasional mid-prophases and practically no late prophases. Therefore the increase of the prophase coefficient in this case seems to mean the increase of the duration of prophase rather than other possibilities. Motion picture sequences of complete mitotic divisions also indicate a short duration for prophase in the early days of incubation. The χ^2 value for the tenth and the twelfth days is also significant. It would seem that the prophase again became shorter. The reason for this is not

clear, but it does not appear to be the effect of the change of the fluid phase medium because first, the χ^2 value between the eighth and the tenth days (two days after a change) is not significant; second, the next change (14 days) gave no demonstrable shift; and finally, the χ^2 between the sixth day and the twelfth day samples is still significant, being 5.606, indicating that even in the twelfth day sample the prophase was significantly longer than at the sixth or earlier days.

TABLE 3

Prophase coefficient of HeLa cultures. Arrows indicate time of changing fluid phase medium.

Days of culture	No. cells in prophase	No. cells in metaphase	Prophase coefficient	χ^2
2	73	282	0.259	
				0.105
4	74	236	0.314	
				0.075
6	82	275	0.298	
				19.832
8	157	258	0.609	
→				0.084
10	179	284	0.630	
				5.804
12	117	284	0.443	
14	77	173	0.445	
→				1.503
16	109	196	0.556	
				0.584
18	93	191	0.487	
20	110	216	0.509	
22	116	261	0.444	
24	105	221	0.475	

Multipolar Spindles. Rapid cell proliferation does not necessarily produce mitotic abnormalities. Embryonic tissues, for instance, exhibit a far lower frequency of abnormal mitoses than many cancer tissues, although both have high rates of cell division. An analysis of the abnormalities can thus be utilized to reveal, perhaps in part, the degree of malignancy. Out of all the mitotic abnormalities the phenomenon easiest for numerical recording and critical analysis is the occurrence of a multipolar spindle, because the numbers of the poles of the spindle during metaphase and anaphase can be counted with reasonable accuracy. From motion picture records it was noted that the number of poles may change during the mitotic course, but this

probably does not seriously affect the sampling because it is likely that these changes compensate for each other. Table 4

TABLE 4

Percentages of the number of poles in metaphasic and anaphasic spindles of HeLa cultures. Arrows indicate time of changing fluid phase medium.

Days of culture	1	2	3	4	5	6	7	8	9	10	>10	Total cells examined
2	1.86	58.36	29.00	7.44	1.49	0.37	0.37	0.37		0.37	0.37	269
4	1.37	67.12	22.74	4.93	1.64	0.55	0.55		0.27	0.27	0.55	365
6	1.48	68.44	21.53	5.31	1.77	0.59	0.29	0.29	0.29			339
8	0.60	91.96	6.25	0.60	0.60							336
→												
10	0.28	90.08	7.71	1.65	0.28							363
12	0.48	91.30	5.80	1.45	0.48	0.48						414
14		90.50	8.26	0.41	0.83							242
→												
16	0.37	88.64	8.42	1.83	0.37	0.37						273
18	0.35	93.75	3.82	1.39		0.35					0.35	288
20	0.32	86.90	10.86	1.60	0.32							313
22		94.00	4.57	1.14		0.29						350
24		92.40	6.08		0.76	0.38					0.38	263

shows the readings for each sample, with number of poles recorded individually up to ten, after which the cells are grouped in the category "more than 10." It is of interest to note that like prophase, the frequency of the two-pole mitotic figures shows a drastic change being greatly increased in percentage by the eighth day of incubation. The χ^2 values comparing the number of the two-poled mitoses and the total cells are presented in table 5. Between the sixth day and the eighth day it is again highly significant, although not as much so as the prophase coefficient. An additional significant value is found in table 5 between the second and the fourth days but there is none between the tenth and the twelfth days.

The Chromosomes. Chromosome counts of HeLa S-17 were made in one set of five-day old cultures. In the 100 cells counted the majority fell into the range 80–100, being more frequent in the 80–90 column (Plate I). Errors in counting would not be expected to exceed ± 3 when the cells contained not more than 100 chromosomes, but would of course be greater for high polyploids. No cell near the diploid number (48) was found, although motion picture records showed the production of small or even

minute daughter cells. This strain probably can be classified as a hypotetraploid.

TABLE 5

2-pole spindles in metaphase and anaphase of HeLa cultures. Arrows indicate time of changing fluid phase medium.

Days of culture	No. cells with two poles	No. cells examined	χ^2
2	157	267	
			4.163
4	245	365	
			0.027
6	232	339	
			6.493
8	309	336	
→			0.355
10	327	363	
			0.006
12	378	414	
14	219	242	
→			
16	242	273	
			0.209
18	270	288	
			0.410
20	272	313	
			0.483
22	329	350	
24	243	263	

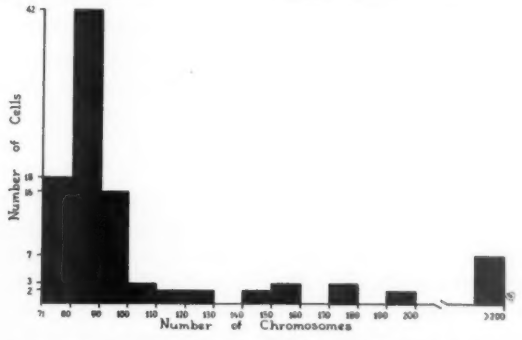


PLATE I. Chromosome number of HeLa S-17, five-day-old sample.

Discussion

The foregoing observations on mitosis of the HeLa strain suggest some interesting points in cancer cytology. They supprt

the conclusion reached by Timonen and Therman that the prophase is short in neoplastic tissues. Probably many abnormalities in the later stages of mitosis result from this due to a lack of synchronization and harmony in the whole mitotic process. Our data have indicated further that after about a week in the same culture medium the mitotic abnormalities became less extreme, the prophase is longer and the multipolar spindles fewer. Whether this is completely due to the nutritional condition is still open to question. Although changes of the fluid phase medium did not restore the abnormalities to their magnitude of earlier days, the nutritional factor cannot be eliminated, because it should be borne in mind that the cultures were still in the original plasma clot. Another possibility is that the subcultures were made from the original cultures by cutting into smaller pieces. This trauma might be a stimulating factor. Experiments are being done to try to clarify these possibilities.

Many of the abnormalities of mitosis are not suitable for quantitative presentation. A mitotic index is probably the most useful criterion for analyses. Pathologists are using it as an aid in determining the degree of malignance. Other workable features include the prophase coefficient, the number of multipolar spindles and the chromosome numbers. In further studies on mitosis in cancer tissues these should all have their places. The chromosome number is probably not a practical criterion for diagnostic purposes because too much work would be involved. The prophase coefficient and the multipolar spindles which easily yield numerical data deserve more attention in diagnosis, and a large scale investigation on pathological specimens regarding these points may be informative indeed.

These factors should be studied in detail for materials that can be maintained *in vitro* or *in vivo* as a constant supply for investigative work. Our data on the chromosome numbers of HeLa only represent the cell population of the fifth day of subculture 17. Many things may affect the chromosome numbers. Since other characteristics have been observed to be changeable when the same set of cultures becomes older, there is no reason to conclude that the chromosomes are not, unless it is supported by substantial data on different age groups of the same set of cultures and from different generations of subcultures. Genetically it is reasonable to suspect the existence of selection forces

applied to a finite population. Probably the living cells are subjected to mutation pressure nearly all the time and roller tube or flask conditions are in a way analogous to those in bacterial cultures and in the population cages of Drosophila where selection forces do apply. As many HeLa workers are aware of the fact that viral infection occasionally destroys stock and as Gey (this symposium) has stated, that the cultural characteristics changed greatly when the stocks were maintained in different media, the selection forces cannot be neglected.

Summary. Cells from a human cervical carcinoma of the HeLa strain in roller tubes with the usual plasma clot and fluid phase medium showed great mitotic activity in the early days of incubation, particularly near the periphery of the cultures. The area near the explant showed a higher mitotic activity after four or five days in culture with the peripheral areas showing rapid drop in cell proliferation. Mitotic division could still be found in considerable numbers even after more than three weeks in the same clot with two changes of the fluid phase medium.

The prophase was short in the earlier days of culture but there was a significant change in the lengthening of prophase after six days.

Multipolar spindles were extremely common in fresh subcultures. Like the prophase, a significant change occurred after six days, with the multipolar spindles being greatly reduced in number.

Most of the cells counted showed chromosome numbers in the range 80–100. The strain can be classified as a hypotetraploid.

Acknowledgments

Acknowledgment is made to Mrs. Mildred Finerty and Mrs. Carolyn Perry for technical assistance.

REFERENCES

Gey, G. O., F. B. Bang, and M. K. Gey: An Evaluation of Some Comparative Studies on Cultured Strains of Normal and Malignant Cells of Animals and Man. *Texas Rep. Biol. and Med.*, 12: 805–827, 1954.

Hsu, T. C.: Tissue Culture Studies on Human Skin. III. Some Cytological Features of the Outgrowth of Epithelial Cells. *Texas Rep. Biol. and Med.*, 10: 336–352, 1952.

————: Mammalian Chromosomes *In Vitro*. IV. Some Human Neoplasms. *J. Nat. Cancer Inst.*, 14: 905–933, 1954.

Hsu, T. C., and C. M. Pomerat: Mammalian Chromosomes *In Vitro*. II. A Method for Spreading the Chromosomes of Cells in Tissue Culture. *J. Hered.*, 44: 23–29, 1953.

Levan, A., and T. S. Hauschka: Endomitotic Reproduction Mechanisms in Ascites Tumors of the Mouse. *J. Nat. Cancer Inst.*, 14: 1–43, 1953.

Scherer, W. F., J. T. Syverton, and G. O. Gey: Studies on the Propagation *In Vitro* of Poliomyelitis Viruses. IV. Viral Multiplication in a Stable Strain of Human Malignant Epithelial Cells (Strain HeLa) Derived from an Epidermoid Carcinoma of the Cervix. *J. Exp. Med.*, 97: 695–710, 1953.

Stilwell, E. F.: Observations of Endomitosis in Embryonic Chick Cells Grown *In Vitro*. *Anat. Rec.*, 114: 9–18, 1952.

Timonen, S., and E. Therman: The Changes in the Mitotic Mechanism of Human Cancer Cells. *Cancer Res.*, 10: 431–439, 1950.

Toolan, H. W.: Growth of Human Tumors in the Subcutaneous Tissues of X-irradiated Laboratory Animals: Their Practical Use for Experimental Purposes. *Cancer Res.*, 12: 302, 1952.

————: Growth of Human Tumors in Cortisone-treated Laboratory Animals: The Possibility of Obtaining Permanently Transplantable Human Tumors. *Cancer Res.*, 13: 389–394, 1953.

STUDIES ON HUMAN CANCER USING SPONGE MATRIX TISSUE CULTURE

I. The Growth Patterns of a Malignant Melanoma, Adenocarcinoma of the Parotid Gland, Papillary Adenocarcinoma of the Thyroid Gland, Adenocarcinoma of the Pancreas, and Epidermoid Carcinoma of the Uterine Cervix (Gey's HeLa Strain)

Joseph Leighton*

Tissue culture methods have been applied to the study of cancer for many years (Coman, 1942 and 1943; Sano and Smith, 1940; Ivers and Pomerat, 1947) but the results have been of limited usefulness to the pathologists, since the morphologic pattern seen *in vitro* usually differed considerably from those seen in microscopic sections of the original tissue. In 1951 it was found that tissues of the chick embryo grew in a combined matrix of cellulose sponge and plasma clot and formed organized aggregates of cells which often made it possible to recognize the tissue of origin. The details of the method have been described (Leighton, 1951). Subsequently it was observed that transplantable tumors of animals could be cultivated in the same way, often with the formation of distinctive tissue patterns in the outgrowth (Leighton, 1953).

In the present report a comparison is made between the microscopic appearance of the original tissue and the growth in culture of five different human tumors. In the first four cases tissue from the recently removed tumors was cut into pieces 1 to 3 mm. in diameter. Some of the fragments were used as explants for culture. Those remaining were fixed and sectioned, and designated as donor fragments. The fifth case, strain HeLa of Gey, was subcultured from cells growing on a glass surface.

---

* From the Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare, Bethesda, Maryland. Presented at the Eighth Annual Cancer Symposium, April, 1954, at the M. D. Anderson Hospital for Cancer Research, Houston.

The nutrient used for the first four tumors consisted of 50 per cent chick-embryo extract, horse serum, and balanced salt solution (Hanks') in the ratio of 1:2:2. One hundred micrograms of penicillin and streptomycin per cubic ml. of nutrient were added. The fifth tumor, the carcinoma of the cervix, was cultivated in commercially prepared HeLa Nutrient.[1]

Malignant Melanoma

Clinical Summary. The patient, a 25-year-old white male, developed a painless mass in his left axilla. Three months later it was excised and diagnosed as metastatic malignant melanoma. He had between 150 and 200 nevi of varying pigmentation, most of them on the trunk. A few were found on the extremities, in the nail beds, in the palms and soles, as well as in the retina, surrounding the anal mucosa, and in the oral mucosa. None appeared to be cancerous, but eight were removed; they were histologically benign. Some were of the junction type.

Eight months later, a mass again appeared in his left axilla. It was excised and part of it was selected for tissue culture. The patient developed progressively severe neurologic symptoms, terminating in coma and death three months after the second operation. Autopsy showed a very large mass of irregularly pigmented tumor tissue completely replacing the left adrenal gland and metastases to the liver, spleen, lymph nodes, and the brain. The primary site was never determined, but it was felt that the tumor arose in the left adrenal or in an unidentified skin lesion.

Donor Tissue. The operative specimen of the axillary tumor contained many firm lymph nodes, some as large as 3 cm. in greatest diameter. Some of the nodes were black; others showed no macroscopic evidence of pigmentation. The tissue selected for culture was from one enlarged, firm node without visible pigment. Microscopic sections of the donor fragments showed metastatic melanoma without pigment, which had almost completely replaced the node. The tumor cells were epithelial-like, large, polyhedral, and closely packed in irregular rows. The cell membranes were distinct, and the cytoplasm stained uniformly with eosin. The nuclei were large and hyperchromatic, with

[1] Microbiological Associates, Bethesda, Maryland.

frequent, large, single, acidophilic nucleoli, or several smaller basophilic nucleoli (Fig. 1). Mitotic figures were present. Sections of most of the tissue used for culture showed no pigment; rare areas in an occasional donor tissue fragment showed a few small macrophages with granules or clumps of brown pigment.

Cultures. Nine cultures were made, incubated at 37.5°C., and fixed for histologic section after three to fifteen days. In cultures kept for more than a week, growth was luxuriant and the explants could no longer be identified. Many epithelial-like tumor cells were found resembling those seen in the donor tissue fragments. Some contained large, acidophilic nucleoli (Fig. 2). In addition, there were many large spindle-shaped tumor cells, often in dense bundles within which small nests of polyhedral melanoma cells were seen (Fig. 3). Mitoses were numerous. Pigment was very prominent, especially as coarse granules of varying size in the cytoplasm of many macrophage cells (Fig. 4). The cytoplasm of some of the tumor cells showed a faint brown hue without distinct pigment granules.

Comment. No diagnostic problem was presented by the mass of enlarged axillary lymph nodes. The donor tissue fragments were non-pigmented, and when seen alone presented a pattern of a poorly differentiated epithelial tumor that could be interpreted as anaplastic carcinoma, hepatoma, or squamous cell carcinoma, as well as melanoma. Under the conditions of tissue culture, a dramatic alteration of the histologic architecture appeared in the new growth. A varied cellular pattern was seen that consisted of spindle-shaped cells, polyhedral tumor cells, and many macrophages containing brown pigment granules.

Adenocarcinoma of the Parotid Gland

(Malignant Mixed Tumor)

Clinical Summary. A 32-year-old white male was admitted following the detection of an asymptomatic mass in his mouth. A smooth, lobulated mass was palpable. It pushed the soft palate and the mucosa of the tonsillar fossa downward and toward the midline, and was also found extending to the angle of the mandible. It was excised and the pathologic diagnosis was "mixed tumor of the parotid."

He was readmitted one year later with enlarged cervical lymph nodes. A resection of the primary area, and a radical neck dissection were performed. Several lymph nodes were found to contain metastases. Tumor tissue adjacent to the parotid salivary gland was submitted for tissue culture. Tumor recurred six months after the second operation.

Donor Tissue. The tumor tissue received for culture was soft, white and mucoid. On microscopic section it was pleomorphic, with small epithelial cells in a variety of patterns (Fig. 5). Cords and masses of cells were found, many with glands of varying size. Some epithelial cell masses were separated from the stroma by a distinct, eosinophilic membrane. Others formed a stellate-celled epithelial reticulum that blended imperceptibly with the cells of the stroma. The stroma, which was abundant, was sparsely cellular, loose, and myxomatous. Rare cartilage-like areas were seen. Part of the specimen was composed of dense fibrous tissue.

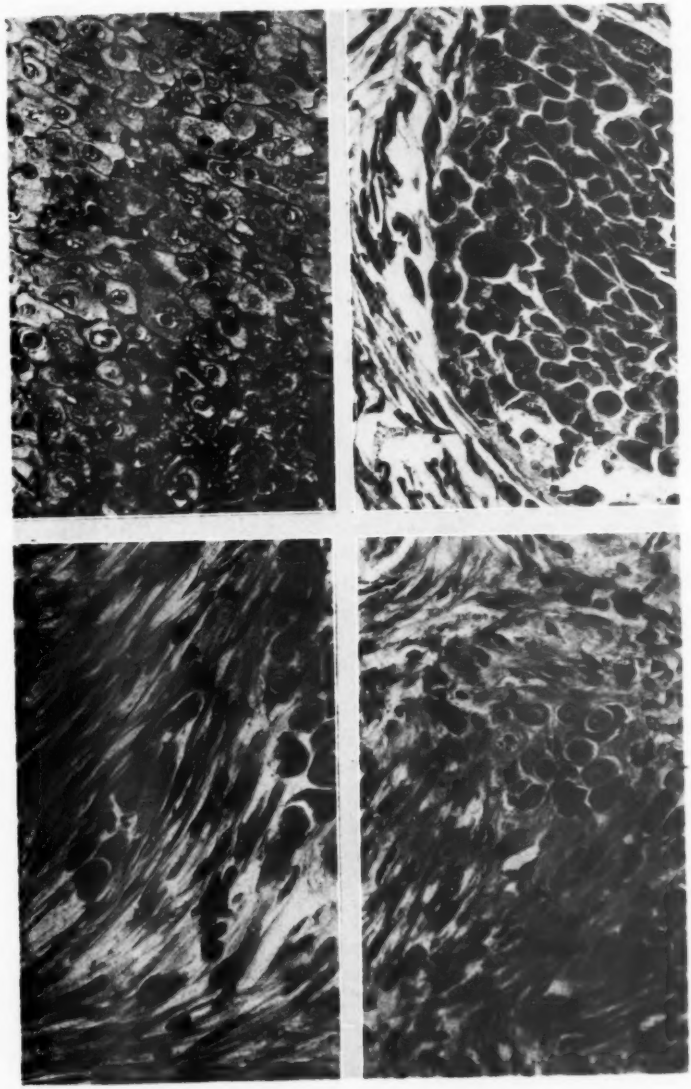
Cultures. Twelve cultures were made, incubated at 35°C., and kept from one to ten days. Growth into the plasma clot was slight. There was progressive flattening and spreading of the explants, and a few epithelial processes gradually extended into the plasma clot. Only a negligible number of spindle-shaped cells grew into the clot. Rapid liquefaction of the plasma clot around the explant may have been partly responsible for the limited outgrowth of all types of cells.

The explants in culture were of interest when compared with sections of the donor tissue fragments. Some explants showed a predominance of fibrous tissue while others were densely cellular (Figs. 6, 7). In the latter interlacing cords and masses of un-

---

FIG. 1. Microscopic section of donor tissue fragment, malignant melanoma, metastatic to a lymph node. The tumor cells are large, and arranged in an epithelial pattern. Prominent acidophilic nuclear inclusions are seen. No pigment is present. Hematoxylin and eosin, X 320.

FIGS. 2, 3, 4. Microscopic sections of tissue cultures of malignant melanoma after 15 days of growth. In figure 2 an epithelial pattern is present, and acidophilic nuclear inclusions can be seen. In figure 3 dense growth of spindle-shaped tumor cells is interspersed with polyhedral tumor cells, singly and in small aggregates. In figure 4 macrophages with abundant brown pigment (black in figure) as well as tumor cells are seen. A pigmented macrophage encircles a tumor cell near the center of the field. Hematoxylin and eosin, X 320.



differentiated epithelial cells were numerous. In parts of these masses, cuboidal epithelial cells formed distinct small glands composed of two concentric cell layers. The cells lining the lumen were cuboidal, the others oval or low-cuboidal. Within a few aggregates of epithelial cells, occasional solitary cells or small groups of cells, showed partial cornification (Fig. 8). These cells were large with abundant cytoplasm that often showed increased eosinophilia. In a few of these groups intercellular bridges could be seen. The stroma was cellular with plump spindle-shaped cells. The volume of extracellular stromal material appeared to be reduced in comparison with the donor fragments.

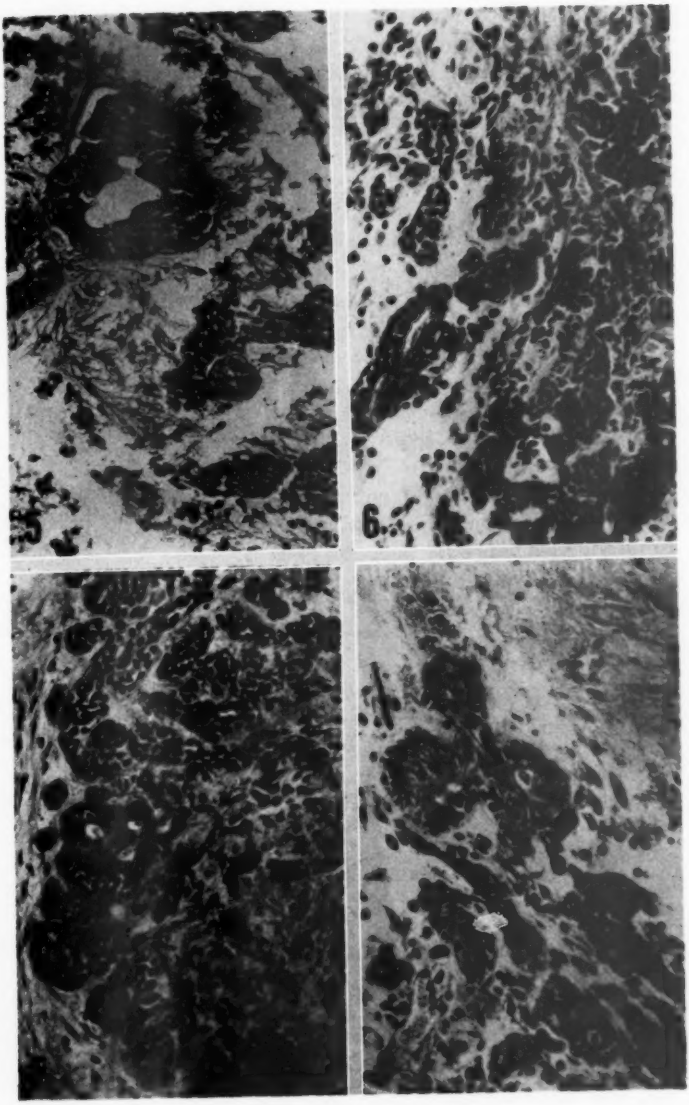
Comment. The predominant proliferation by epithelial cells seen here is consistent with other reports on tumors of this type in tissue culture (Zymbal, 1933; Cameron and Chambers, 1937; Favata, 1948). No cartilage-like areas developed, nor was the varied appearance of a teratoma seen. Cartilage grows very slowly *in vitro* and its failure to develop during a ten day period of cultivation does not preclude the possibility of its ultimate appearance. The results in these cultures, nevertheless, are consistent with the concept that "malignant mixed tumors" of the parotid are of epithelial origin and not of mixed cellular origin.

Papillary Adenocarcinoma of the Thyroid Gland

Clinical Summary. A 26-year-old white male was admitted complaining of a mass in the right side of his neck for six months, and one on the left side for three months. Biopsy of a right cervical lymph node showed metastatic papillary cystadenocarcinoma of the thyroid gland. Bilateral radical neck dissections and bilateral hemithyroidectomy were performed in two stages.

FIG. 5. Microscopic section of donor fragment, adenocarcinoma of the parotid gland. Groups of poorly differentiated epithelial cells are seen. Some epithelial masses form glandular structures. The stroma is sparsely cellular and mucinous. Hematoxylin and eosin, X 250.

FIGS. 6, 7, 8. Microscopic sections of explants of parotid carcinoma in tissue culture for ten days. In comparison with donor fragments there is increased cellularity, especially of epithelial cells, which form thick anastomosing cords. In some areas, glands are formed consistently having a wall composed of two cell layers. Cornification is found in occasional groups of large cells (indicated by an arrow). Hematoxylin and eosin, X 250.


5
6

On pathologic examination the primary tumor was found in the right half of the thyroid gland. Vascular invasion was present. Many of the removed cervical lymph nodes contained tumor. A piece of a node was obtained for tissue culture. The patient was well, and without evidence of recurrence one year after discharge.

Donor Tissue. The lymph node on section was tan, with yellow and purple areas. In cutting explants, 1.5 mm. in diameter, portions of the node were found to be fibrous and cut with difficulty, while other areas were gelatinous. Five of fourteen donor tissue fragments contained tumor (Fig. 9). The others appeared normal or showed reticular hyperplasia, or fibrosis.

The foci of tumor appeared as microcystic structures which were lined by neoplastic columnar epithelium. Groups of tumor cells were often arranged in papillary processes extending into the cyst spaces, sometimes in contact with the tips of other papillary groups. Where this pattern was extensive, tumor cells in a loose trabecular arrangement filled the cyst space. In some cysts, basophilic laminated calcific bodies were seen. No follicles containing colloid could be found.

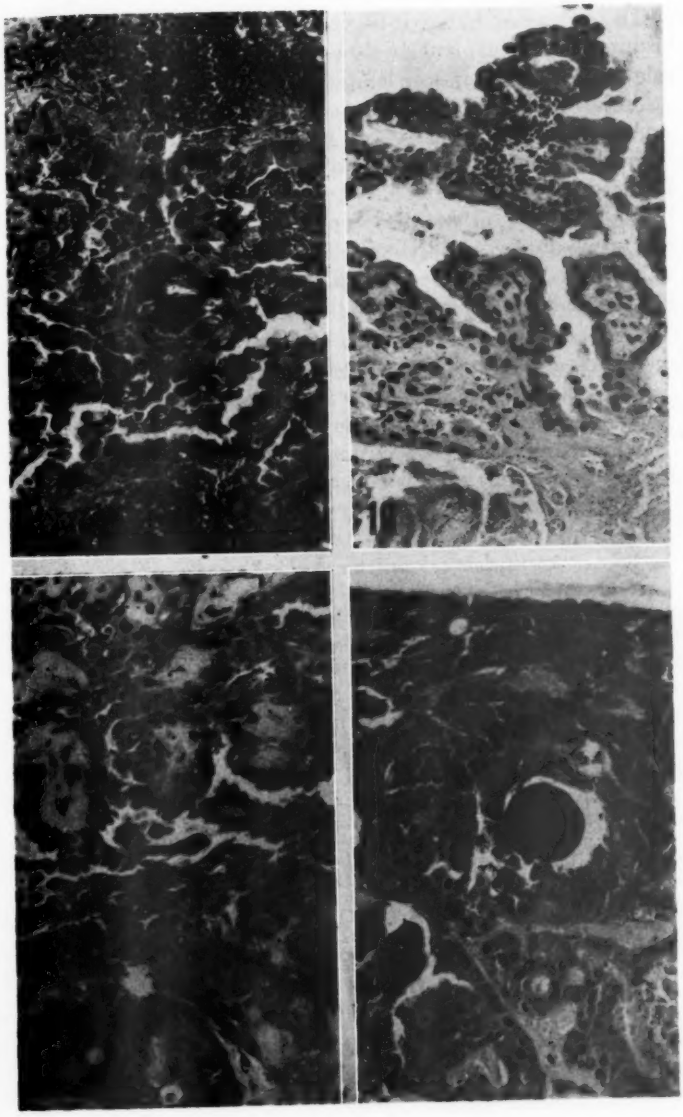
Cultures. Twelve cultures were made. Six were incubated at 37.5°C., the others at 35°C. Cultures were fixed from 7 to 22 days after preparation. Outgrowth from the explants, luxuriant in some cultures, was limited to spindle-shaped cells and round cells. However, tumor tissue was found within some of the explants. The ratio of explants containing tumor to total explants was the same in culture as in the donor tissue fragments examined.

FIG. 9. Microscopic section of a donor tumor fragment, papillary adenocarcinoma of the thyroid, metastatic to a cervical lymph node. The arrangement of the carcinoma cells, and a calcific body are shown. Hematoxylin and eosin, X 250.

FIG. 10. Microscopic section of an explant of thyroid tumor in tissue culture after one week. A papillary pattern is present. Degenerating cells are seen in the stromal core of a papillary process. Hematoxylin and eosin, X 250.

FIG. 11. Microscopic section of an explant of thyroid tumor in tissue culture after three weeks. Tumor cells form cords and glands as well as papillary patterns. Hematoxylin and eosin, X 250.

FIG. 12. Microscopic section of an explant of thyroid tumor in tissue culture after three weeks. Calcific bodies are seen as black laminated or fragmented structures. Some tumor cells are in intimate contact with intercellular eosinophilic hyaline material, indicated by arrows. Hematoxylin and eosin, X 250.



The epithelial tumor cells varied in different explants, and within the same explant. In three cultures, a pattern of papillary adenocarcinoma was seen (Figs. 10, 11). Hyaline, eosinophilic material, possibly colloid, was seen in some glandular lumens and in some interstitial spaces. Calcified basophilic concretions were also found (Fig. 12). One cannot say definitely on the basis of these cultures whether these concretions were all part of the original explant, or whether some have formed *in vitro*. Tumor was seen equally well in explants fixed from 8 to 22 days after cultivation. In the poorest tumor explants, ghost forms of tumor cells could be recognized, as well as occasional calcific bodies.

Comments. Since the number of cultures was small, and only one third of the explants showed tumor tissue, no conclusion can be reached regarding the effect of incubation at 35°C. as compared with 37.5°C. It is clear that since only spindle-shaped cells and round cells grew out of the explants, one could not be certain that tumor cells were present in the cultures until the explants themselves had been examined in histologic sections.

Adenocarcinoma of the Pancreas

Clinical Summary. A 64-year-old white grocer complained of lower-back pain for six months. During the same period flatulence, indigestion, and shortness of breath were present. On admission, there was fluid in the left chest, the liver was enlarged 3 cm. below the costal margin, and a solitary, hard nodule in the prostate was palpable. Extensive X-ray studies demonstrated a fluid level up to the eighth rib on the left, obliteration of the right costophrenic sinus, and bilateral interstitial pulmonic infiltration. A gastrointestinal series showed partial obstruction near the ligament of Treitz.

A thoracentesis was performed, part of the fluid being sent to the cytology laboratory and some being submitted for tissue culture studies. The cytology laboratory reported: "Papanicolaou Class II; Cellular debris, no tumor cells identified."

The patient was discharged from the hospital a few days later at his own request. During the period of hospitalization several diagnoses were considered. These were cancer with metastases from a primary tumor in the pancreas, prostate, kidney, or lung. The patient died two weeks after leaving the hos-

pital. An autopsy showed adenocarcinoma of the body and tail of the pancreas with many metastases.

Donor Tissue. A 400 cc. volume of straw-colored fluid contained a floating white coagulum 4 cm. in diameter. The coagulum was removed, gently kneaded to reduce its volume, and areas with a granular, opaque appearance were selected for study. These were cut into fragments and treated as tissue. Histologic sections of the donor clot fragments showed an eosinophilic coagulum with degenerating cells, leucocytes, individual large cells with prominent nuclei, and clusters of vacuolated epithelial-like cells suggesting neoplastic glandular epithelium (Figs. 13, 14).

Cultures. Four cultures were made from clot fragments. A fifth culture was prepared from the cell sediment obtained on centrifugation of 200 ml. of the remaining fluid. All were incubated at 35°C., and fixed 5 to 21 days after preparation.

Sections of a culture after five days of incubation showed an outgrowth of many spindle-shaped, as well as round cells into the interstices of the sponge. The fragments of explanted coagulum were intact, and intensely eosinophilic in histologic preparations. Within the explanted clot particles were many large tumor cells, some single and others in groups. These latter often appeared in distinct acinar patterns.

After 9 days there was partial disruption of the explanted coagulum fragments by many irregular fissures. Most of the tumor epithelial cells were still within the substance of the explanted coagulum, although a few could also be found in the sponge interstices. Glandular structures were numerous (Figs. 15, 16).

After 21 days, there were marked changes in the explanted coagulum. It was fragmented by many intersecting spaces in which spindle-shaped cells, round cells, and a few multinucleate giant cells were found. Complex glandular aggregates of tumor epithelium were usually in contact with bundles of proliferating spindle-shaped cells and occasionally with sponge trabeculae (Figs. 17, 18, 19).

Comment. The large cells, found on examination of histologic sections prepared from the pleural fluid coagulum before cultivation, made the diagnosis of carcinoma fairly sure, although there was some difference of opinion. Some felt that no definite tumor cells could be identified, and that the large epithelial-

like cells in the coagulum were possibly of pleural (mesothelial) origin. Examination of a few sections of the cultures showed definite neoplastic glandular epithelium, making the diagnosis of adenocarcinoma a certainty.

Epidermoid Carcinoma of the Uterine Cervix (HeLa)

Background. In February, 1951, Gey, Coffman, and Kubicek (1952) succeeded in cultivating a strain of tumor cells from an epidermoid carcinoma of the cervix which they designated as strain HeLa.

A section of the original tissue specimen from which the culture strain was derived is of interest.[2] The tumor tissue consisted of a poorly differentiated carcinoma which was diffusely infiltrating connective tissue. Mitotic figures were very numerous. The tumor cells appeared singly, and in irregular masses. The pattern of the cells, especially in areas with thick branching cords of cells, was characteristic of epidermoid carcinoma. More differentiated features of epidermoid carcinoma, such as keratinization or the presence of intercellular bridges, were not seen (Fig. 20).

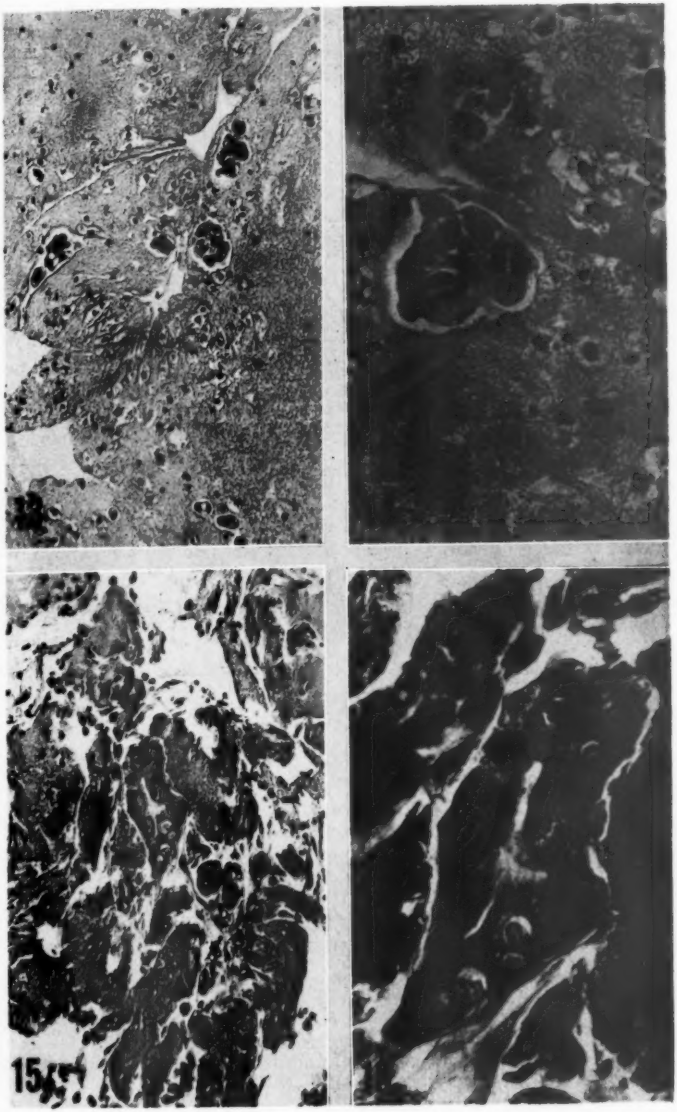
The tumor has been grown on the glass wall of culture tubes in a variety of nutrient mixtures most of which contain adult human serum, or human placental cord serum as a basic component. The cells have since been used in the study of virus infections by Scherer, Syverton, and Gey (1953).

Donor Tissue. HeLa cells grow on the bare wall of culture tubes, or on thin plasma clots, with great variation in size and shape often in the same microscopic field. The cells may be kite-shaped, spindle-shaped, or round (Fig. 21). As growth increases, the cells form a continuous membrane that appears as a mosaic

[2] I am very grateful to Dr. George O. Gey of the Johns Hopkins Hospital for lending me a section of this specimen.

FIGS. 13, 14. Microscopic section of donor fragments of pleural fluid clot from patient with carcinoma of the pancreas. Degenerating cells, leucocytes and large cells, probably neoplastic, singly and in clusters are seen. Hematoxylin and eosin, X 200, X 500.

FIGS. 15, 16. Microscopic section of a nine-day-old culture showing the explant of pleural fluid clot. Several groups of neoplastic epithelial cells are seen including a glandular structure. Hematoxylin and eosin, X 200, X 500.



on microscopic examination. Small clumps of HeLa cells were obtained by scraping the wall of a tube in which a sheet of tumor cells was growing. The cell clumps were used immediately as explants for sponge cultures.

Cultures. HeLa cells have been grown in cellulose sponge at 35°C. and at 37.5°C. Growth was vigorous, and after four to six weeks the cultures required patching with fresh plasma and a complete change of nutrient every day. After about eight weeks, the nutritional needs of the tumor tissue exceeded the nutrient supply available in the roller tube system, 1.5 cc. per tube, and the cultures were interrupted. The pH of the HeLa nutrient mixture changed from 7.6 to 6.8 or lower in less than twenty-four hours. Many thick cords and masses of tumor cells could be seen within the sponge interstices.

Histologic sections of the sponge showed a poorly differentiated carcinoma. Mitotic figures were very numerous, and some were multipolar. Small round areas of necrosis of tumor cells were seen (Fig. 22). In an occasional area barely visible protoplasmic fibrils could be found between the surfaces of tumor cells, but they did not have the regularity usually seen in the intercellular bridges of squamous epithelium (Fig. 23, see arrow).

Comment. After two years of growth *in vitro*, when taken from the glass wall of a tube and grown in a sponge, this tumor retained the appearance of a poorly differentiated epidermoid carcinoma.

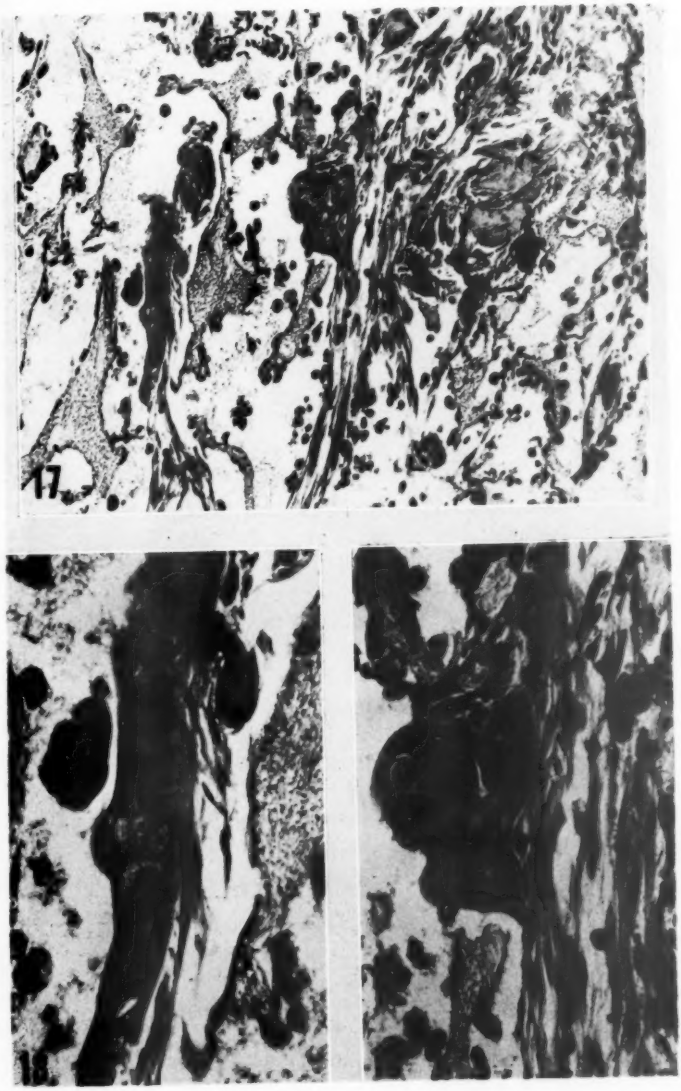
Discussion

In this study, as in previous ones using tissues from the chick embryo and transplantable animal tumors, the potentialities of many kinds of cells for forming organized aggregates of cells *in vitro* appear to be enhanced by the distinctly three dimensional environment of the sponge interstices. Dense masses

---

FIG. 17. Microscopic section of a 21-day-old culture of pleural fluid. The donor coagulum has almost completely dissolved and spindle-shaped cells, round cells, and groups of neoplastic, glandular, epithelial cells are seen. Hematoxylin and eosin, X 200.

FIGS. 18, 19. Details of two epithelial structures from figure 17. X 500, X 400.



of cells may grow within the sponge spaces and form tissues. When sponge cultures are examined with histologic methods, new tissue is seen that has many of the structural qualities of tissue from the body, and readily lends itself to study within the frame of reference of pathologic anatomy.

The method may provide a supplement to the methods used by the surgical pathologist in the study of tumors. There are two practical clinical areas where it may be immediately applied, first, as a method in the diagnostic examination of ascites fluid and pleural effusions, second, as a supplementary aid in the histogenic classification of tumors.

In addition it suggests a vehicle for direct experimental examination of human cancer tissue with possibility of a good correlation of the observations made *in vitro* with those made on the patient.

Summary. Five human tumors have been cultivated *in vitro* using the sponge matrix method.

Areas of non-pigmented metastatic malignant melanoma in an axillary lymph node produced abundant pigment in culture.

An adenocarcinoma of the parotid showed marked proliferation of epithelium within the explants.

A cervical lymph node with metastases from a papillary adenocarcinoma of the thyroid showed a papillary glandular structure on the surface of several tumor explants.

A diagnostic problem, a pleural effusion, believed to be due to metastatic cancer, primary site undetermined, gave rise to many glandular structures after five days in culture. Adenocarcinoma of the pancreas was found subsequently at autopsy.

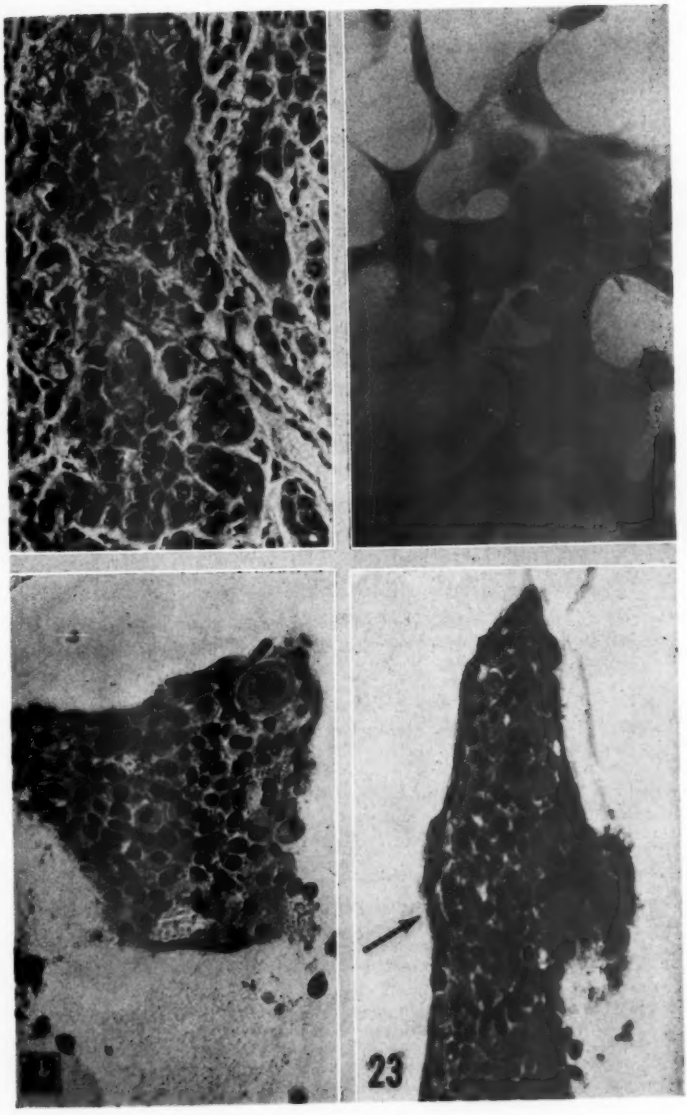
Cells from a carcinoma of the uterine cervix, strain HeLa of Gey, which has been kept on a glass surface since February, 1951, grew in sponge matrix as thick irregular masses and cords,

---

FIG. 20. Microscopic section of original surgical specimen (strain HeLa), a poorly differentiated epidermoid carcinoma of the cervix, placed in tissue culture by Dr. George O. Gey on February 8, 1951. Hematoxylin and eosin, X 320.

FIG. 21. HeLa cells growing on a bare glass surface as a perforated membrane, one cell layer thick. Hematoxylin and eosin, X 320.

FIGS. 22, 23. Microscopic sections of a one-month-old culture of HeLa. The cells form compact masses and have an epidermoid pattern. Intercellular bridges are suggested in some areas as indicated by the arrow. Dividing cells are numerous. Hematoxylin and eosin, X 320.


23

and on section of the cultures appeared to be a poorly differentiated epidermoid carcinoma.

Acknowledgments

I wish to acknowledge the assistance of the following persons who contributed the tumor tissues used in this study: Dr. L. L. Ashburn, Public Health Service Hospital, Baltimore, Maryland (Malignant Melanoma); Dr. Calvin T. Klopp, the George Washington University School of Medicine, Washington, D. C. (Adenocarcinoma of the Parotid Gland, and Papillary Adenocarcinoma of the Thyroid Gland); Dr. Benjamin Manchester and Dr. Ivor Cornman, the George Washington University School of Medicine, Washington, D. C. (Adenocarcinoma of the Pancreas); Dr. William F. Scherer, University of Minnesota Medical School, Minneapolis (Epidermoid Carcinoma of the Uterine Cervix, Strain HeLa of Gey).

REFERENCES

Cameron, G., and R. Chambers: Neoplasm Studies. III. Organization of Cells of Human Tumors in Tissue Culture. *Am. J. Cancer*, 30: 115–129, 1937.

Coman, D. R.: Human Neoplasms in Tissue Culture. *Cancer Research*, 2: 618–625, 1942.

————: Human Neoplasms in Tissue Culture. II. Observations upon Cells Derived from Peritoneal and Pleural Effusions. *Cancer Research*, 3: 526–530, 1943.

Favata, B. V.: Characteristics of Mixed Tumors of the Parotid Gland Growing *In Vitro*. *Surg. Gynec. and Obst.*, 86: 659–662, 1948.

Gey, G. O., W. D. Coffman, and M. T. Kubicek: Tissue Culture Studies of the Proliferative Capacity of Cervical Carcinoma and Normal Epithelium. *Cancer Research*, 12: 264–265, 1952.

Ivers, J. B., and C. M. Pomerat: Observations on Cells from Ascitic Fluid Cultured *In Vitro*. *Texas Rep. Biol. and Med.*, 5: 92–111, 1947.

Leighton, J.: A Sponge Matrix Method for Tissue Culture. Formation of Organized Aggregates of Cells *In Vitro*. *J. Nat. Cancer Inst.*, 12: 545–561, 1951.

————: A Survey of the Growth *In Vitro* of Representative Types of Transplantable Animal Tumors Using the Sponge Matrix Method for Tissue Culture. *Anat. Rec.*, 115: 453, 1953.

Sano, M. E., and L. W. Smith: Tissue Culture as Diagnostic Aid in Identification of Atypical Tumors. *Arch. Path.*, 30: 504–508, 1940.

Scherer, W. F., J. T. Syverton, and G. O. Gey: Studies on the Propagation *In Vitro* of Poliomyelitis Viruses. IV. Viral Multiplication in a Stable Strain of Human Malignant Epithelial Cells (Strain HeLa), Derived from an Epidermoid Carcinoma of the Cervix. *J. Exp. Med.*, 97: 695–710, 1953.

Zymbal, W. E.: Histologische und experimentelle Untersuchungen über die Geschwülste der Speicheldrüsen. *Beitr. z. Path. Anat. u. z. allg. Path.*, 91: 113–175, 1933.

STUDIES ON HUMAN CANCER USING SPONGE MATRIX TISSUE CULTURE

II. Invasion of Connective Tissue by Carcinoma (Strain HeLa)*

Joseph Leighton† and Ira Kline‡

This report concerns an approach to the study of the invasiveness of human cancer using the sponge matrix method for tissue culture (Leighton, 1951).

Many studies on cancer *in vitro* have considered the structure and functional behavior of neoplastic tissue in terms of the morphologic and metabolic characteristics of the tissue in which the tumor arose. Endocrine functions, and the formation of mucin, keratin, and melanin have been described. The quality of cancer, however, which distinguishes it critically from its tissue of origin is its malignancy, *i.e.*, the ability of cancer to grow, invade, and seriously alter, or almost entirely replace normal tissue, and its ability to release minute metastases with the same properties. Difficulties of technique, and of recognition of cell types may have discouraged the widespread use of *in vitro* methods in the study of tissue interaction. Nevertheless Santesson (1935), Ludford and Barlow (1944), and Fischer (1929, 1930) have examined the interaction of mouse mammary tumors and connective tissue.

In the experiments described here, small clumps of human carcinoma cells were transplanted into established cultures of human connective tissue to examine the pattern of interaction of the two. The connective tissue was derived from the foreskin of 4-day-old infants; the carcinoma cells were derived from carcinoma of the cervix, strain HeLa (Gey, Coffman and Kubicek, 1952; Scherer, Syverton and Gey, 1953).

---

* From the National Cancer Institute, National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare, Bethesda, Maryland. Presented at the Eighth Annual Cancer Symposium, April, 1954, at the M. D. Anderson Hospital for Cancer Research, Houston.

† Laboratory of Pathology, National Cancer Institute.

‡ Laboratory of Chemical Pharmacology, National Cancer Institute.

Observations

First Series. Ten cultures of connective tissue were prepared. Three explants of foreskin, each between 1 and 2 mm. in diameter, were placed upon thin slices of cellulose sponge in each of nine roller tubes. The slice of sponge in the tenth tube was prepared with a hole 5 mm. in diameter punched in the center. Two explants were placed on the circumference of the hole. A clotting mixture of heparinized chicken plasma and "liquid nutrient" was used. The "liquid nutrient" was composed of horse serum, Hanks' balanced salt solution, and a 50 per cent extract of 11-day-old chick embryos in a ratio of 2:2:1. An antibiotic content of 50 micrograms/ml. of streptomycin and penicillin G was maintained in the nutrient. The tubes were incubated at 35°C., in a roller drum. "Liquid nutrient" was replaced as needed, usually two or three times each week.

In two weeks the growth of spindle-shaped cells was extensive around many explants. After 32 to 38 days of cultivation of the connective tissue, barely visible clumps of tumor cells were cemented with a thin plasma patch to the five foreskin cultures showing the most luxuriant growth. One of these was in the sponge with the hole, where the new connective tissue had completely filled the space. The tumor cells had been grown, up to this time, for many transplant generations, on the bare glass wall of roller tubes in a nutrient of 40 per cent human placental serum. Clumps of tumor cells were obtained by scraping the wall of the tube with a pipette. The liquid medium used in the combined cultures was a mixture of equal volumes of "liquid nutrient" and 40 per cent human placental serum.

Growth of the tumor cells into the connective tissue, and its apparent replacement by tumor, was evident in the daily examination of each of the living cultures. Tumor and connective tissue cells could often be distinguished. The fibroblasts were small and spindle-shaped; the tumor cells were large, globular, and relatively refractile. In two cultures the small inoculum of tumor cells could not be identified immediately after preparation, since the tumor cells were obscured by the sponge as well as by the connective tissue. However, after four days, tumor cells were seen in large numbers. The culture with the central hole was studied for 13 days after inoculation with tumor. In-

filtration and replacement of the dense connective tissue growth by enormous numbers of closely packed carcinoma cells was obvious. The combined cultures after 7 to 10 days required a daily change of liquid nutrient.

Six to 13 days after the addition of tumor cells the cultures were fixed in Zenker's formalin for serial sectioning. In sections, whenever connective tissue and tumor were in direct contact the cancer infiltrated the connective tissue cells. The appearance of these areas confirmed the observations on the living cultures. The connective tissue appeared to be undergoing dissolution in the presence of the tumor. The tumor cells showed some variation in appearance in every field. In areas where tumor cells were densely packed and spindle cells were almost absent, small foci of punctate necrosis were often seen. The viable tumor cells contained relatively small, densely staining nuclei and scant cytoplasm. In other areas, usually where tumor and connective tissue cells were in intimate contact, the tumor cells were often large and their cytoplasm was abundant. Nuclei here were very large and contained one or two large prominent basophilic nucleoli. Normal and multipolar metaphases were numerous throughout the areas of tumor cells. The wide range seen in the size of tumor cells is shown in figures 5, 6, 7, and 8, which were all made at the same magnification, X 320.

Second Series. The experiment was repeated in a second series. Here the liquid nutrient used during the period of interaction was composed of 10 parts of Hanks' balanced salt solution, 5 parts of pooled adult human serum, and 1 part of a 50 per cent extract of beef embryos, and contained 100 micrograms of penicillin and streptomycin per c.c. of nutrient.

It was considered desirable to identify the site of tumor implantation in histologic sections to obtain a better index of the extent of tumor outgrowth. Since gelatin sponge[1] is histologically different from cellulose sponge, the method of cultivating the stock of HeLa cells was changed from growth on a glass surface to growth in a matrix of Gelfoam particles. It was hoped that particles of Gelfoam containing tumor could be added easily to

[1] Gelfoam, The Upjohn Co., Kalamazoo, Mich.

the connective tissue cultures, and subsequently be identified in histologic sections.

Dry Gelfoam was moistened with liquid medium and minced with scissors into particles approximately 1 mm. in diameter. When the suspended Gelfoam particles were added to a culture of HeLa on glass, the cells became attached to the gelatin sponge particles in 24 to 48 hours, and vigorous proliferation continued. With a little practice, cells could be easily recognized in the interstices of the gelatin sponge particles in the living cultures. Gelfoam digestion was slight, and additional particles were added from time to time as needed. Liquid nutrient was changed as indicated by the pH of the medium. Subcultures were prepared by pipetting tumor-bearing Gelfoam particles into tubes with freshly prepared particles and liquid nutrient.

Microscopic sections of HeLa in Gelfoam particle cultures showed many aggregates of rounded tumor cells (Fig. 2). Mitoses were very frequent. The gelatin sponge is amphophilic to hematoxylin and eosin, and has a homogenous hyaline appearance.

Fragments of infants' foreskin were cultivated in six tubes using slices of cellulose sponge with holes. In five tubes there was excellent growth of connective tissue cells, completely filling the hole in one month. Small Gelfoam particles containing tumor cells were added to four 35-day-old connective tissue cultures. The inoculum was placed at the rim of the hole in the sponge, preferably in a pocket of clot liquefaction, and a thin plasma patch was added (Fig. 3).

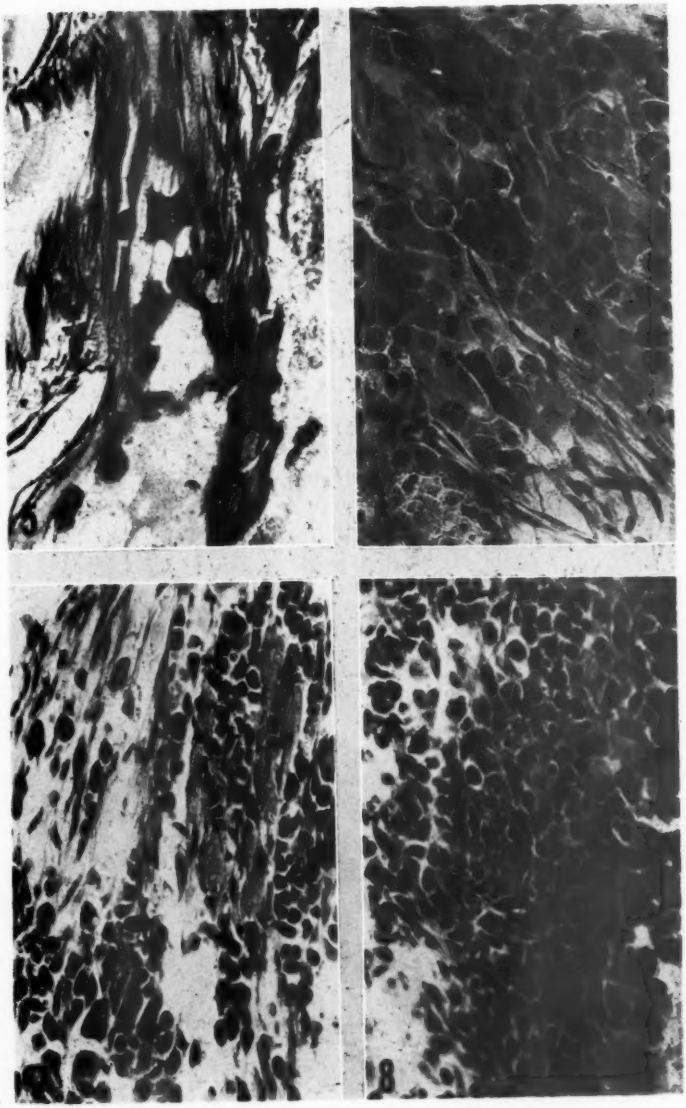
Each of the tubes showed the same pattern as did the first series, and the progress of the tumor in the living cultures could be followed closely within the hole in the sponge. In 3 or 4 days,

---

FIG. 5. Microscopic section of a culture 9 days after the addition of tumor. Connective tissue cells are invaded by kite-shaped tumor cells. Hematoxylin and eosin, X 320.

FIG. 6. Microscopic section of a culture 13 days after the addition of tumor. The field is from the center of the punched out area and shows large tumor cells infiltrating spindle-shaped connective tissue cells. Hematoxylin and eosin, X 320.

FIGS. 7, 8. Microscopic section of a culture 21 days after the addition of tumor. In figure 7, many small tumor cells are infiltrating the connective tissue. In figure 8, from an adjacent field, connective tissue has been almost entirely replaced by a compact mass of tumor cells. Hematoxylin and eosin, X 320.



individual tumor cells had migrated short distances into the connective tissue. After two weeks the zone of interaction of connective tissue and tumor was very broad, and had moved into the area previously occupied only by connective tissue (Fig. 4). After 3 weeks the connective tissue areas were further and markedly reduced, and replaced by tumor tissue. Aggregates of packed tumor cells, following in the wake of the zones of interaction, showed little intercellular cohesiveness, and floated off into the liquid medium. Two cultures were fixed after 14 days and two after 21 days. Histologic sections presented the same patterns as did the first series. The areas of tumor inoculation were identified by the residual Gelfoam.

The single connective tissue culture that did not receive a tumor inoculum was fixed after 49 days. On microscopic examination areas of densely and sparsely cellular connective tissue outgrowth were seen (Fig. 1).

At the same time that HeLa in gelatin sponge was added to the four connective tissue cultures, two control cultures of tumor were prepared. Particles of Gelfoam containing tumor were planted on the edge of holes in slices of cellulose sponge and a plasma clot was added. In marked contrast to the outgrowth of comparable tumor inocula into connective tissue, these showed almost no proliferation during the three-week period of observation.

Discussion

Santesson (1935) studied the interaction of mouse mammary tumors of varying clinical malignancy with embryonic con-

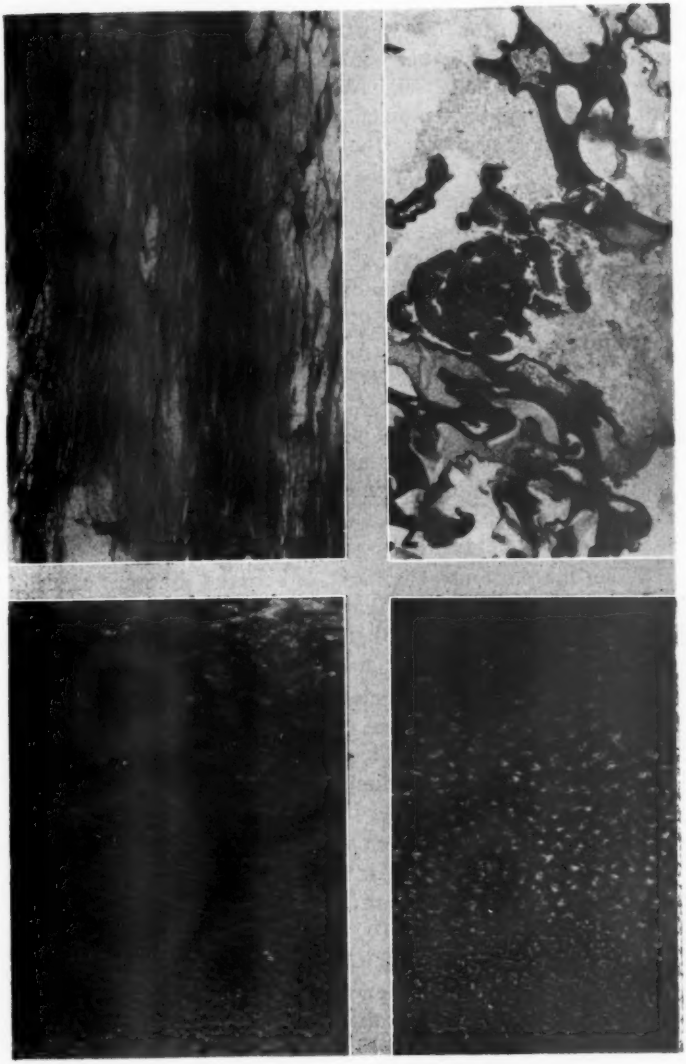
---

FIG. 1. Microscopic section of a culture of connective tissue after 49 days of growth. Hematoxylin and eosin, X 320.

FIG. 2. Microscopic section of a culture of HeLa showing an aggregate of tumor cells growing in a matrix of Gelfoam particles in fluid suspension. Hematoxylin and eosin, X 320.

FIG. 3. A living culture showing the line of contact between connective tissue and tumor after 1 day. On the previous day a particle of Gelfoam containing tumor cells had been transplanted into a 35-day-old culture of connective tissue. A spicule of cellulose sponge conveniently points to the border of the two tissues. The tumor in Gelfoam is in the upper part of the illustration, and the connective tissue is in the lower part, X 150.

FIG. 4. The living culture of connective tissue seen in figure 3, 13 days after receiving the tumor transplant. The tumor cells are globular and refractile, resembling sago granules. In this area as in all zones of contact between tumor and connective tissue the tumor invades and replaces the connective tissue cells over broad zones of interaction, X 150.



nective tissue. He observed that the most malignant tumors tended to infiltrate the connective tissue diffusely while the benign tumors frequently became encapsulated by the growing connective tissue cells. We have seen a highly malignant human tumor infiltrating and replacing connective tissue cells in sponge matrix, and are interested in learning something about the mechanisms of this interaction and the possibility of producing fibrous encapsulation of this tumor in culture.

Ludford and Barlow (1944) described the influence of several mouse tumors on embryonic connective tissue. Some tumors stimulated and others inhibited the growth of connective tissue cells. Since we implanted a small tumor inoculum into a dense culture of connective tissue, we are unable to recognize stimulation or inhibition of the connective tissue by the tumor.

In the studies of Fischer (1929, 1930) cited by Ludford and Barlow, marked stimulation of the growth of mouse mammary cancer cells was obtained by adding explants of embryonic connective tissue. In our experiments, small clumps of HeLa cells grew much more rapidly in combination with connective tissue, than when cultivated alone.

When the patterns of interaction between different kinds of human tissues in culture have been reliably characterized, some of the elements of malignant phenomena in man will be accessible for direct biologic and therapeutic study.

The use of dispersed particles of gelatin sponge, suspended in a liquid medium, as a nutrient and a physical support for growth made the cultivation of large quantities of HeLa cells in small aggregates and as individual cells technically simple and practical.

Summary. A dense growth of connective tissue cells was obtained from explants of infant's foreskin, after five weeks of cultivation in a combined matrix of cellulose sponge and plasma clot.

Cancer cells (Strain HeLa) derived from a carcinoma of the cervix were cultivated both on the bare glass surface of a roller tube as heretofore, and in the interstices of Gelfoam sponge particles that were suspended in a liquid nutrient.

Clumps of tumor cells scraped from the glass wall, or particles of Gelfoam containing tumor cells, were placed upon the con-

nective tissue. With either tumor inoculum the tumor cells grew, infiltrated, and replaced the connective tissue extensively.

Acknowledgments

We wish to acknowledge the help of Dr. John L. Parks of the George Washington University School of Medicine, Washington, D. C., who made available the normal tissue used in the study. Grateful acknowledgment is made to Miss Zelda Dorin for technical assistance.

REFERENCES

Fischer, A.: Gewebezüchtung: *Handbuch der Biologie der Gewebezellen In Vitro*. 3rd ed. Mueller, Munich, 1930.

Fischer, A., H. Laser, and H. Meyer: Wechselbeziehungen swischen normalen und bösartigen Geweben. *Ztschr. f. Krebsforsch.*, 29: 270–301, 1929.

Gey, G. O., W. D. Coffman, and M. T. Kubicek: Tissue Culture Studies of the Proliferative Capacity of Cervical Carcinoma and Normal Epithelium. *Cancer Research*, 12: 264–265, 1952.

Leighton, J.: A Sponge Matrix Method for Tissue Culture. Formation of Organized Aggregates of Cells *In Vitro*. *J. Nat. Cancer Inst.*, 12: 545–561, 1951.

Ludford, R. J., and H. Barlow: The Influence of Malignant Cells upon the Growth of Fibroblasts *In Vitro*. *Cancer Research*, 4: 694–703, 1944.

Santesson, L.: Characteristics of Ephithelial Mouse Tumor Cells *In Vitro* and Tumor Structures *In Vivo*. A Comparative Study. *Acta Path. et Microbiol. Scand., Suppl.* XXIV, 1–237, 1935.

Scherer, W. F., J. T. Syverton, and G. O. Gey: Studies on the Propagation *In Vitro* of Poliomyelitis Viruses. IV. Viral Multiplication in a Stable Strain of Human Malignant Epithelial Cells (Strain HeLa), Derived from an Epidermoid Carcinoma of the Cervix. *J. Exp. Med.*, 97: 695–710, 1953.

THE USE OF TETRAZOLIUM SALTS FOR SHORT TERM TISSUE CULTURES OF SURGICAL SPECIMENS

PETER M. MARCUSE*

This paper represents the continuation of studies on the effect of neotetrazolium on cells grown *in vitro* from human biopsy material. A previous report on this subject was based on a series of 70 cases in which the cells of the out-growths had been examined for their reaction to a neotetrazolium-succinate mixture (Marcuse, 1954). It was found that the resulting formazan deposits could be divided into the crystal type and the droplet form, the crystalline precipitate probably being the more significant indicator of intracellular reductase activity.

The present series is based on out-growths from 88 specimens of which 58 were used for observation of their reaction with neotetrazolium. None of the previously reported cases were used for this study which comprised 34 benign specimens and 24 malignant tumors. Neotetrazolium-succinate mixture was applied to the out-growths in the previously reported manner and the results were tabulated according to the same criteria. In addition, however, several staining methods were employed in an attempt to solve problems which are encountered in using tetrazolium salts on tissue cultures. These particular difficulties are not met with in working with tissue slices or homogenates, and their solution can, therefore, not be found in the voluminous literature which is available on the application and effect of the tetrazolium compounds but includes very little information on their use in the field of tissue culture.

Material and Methods. The tissue culture method was the hanging drop procedure which had been described previously (Marcuse, 1954). The final penicillin concentration, however, was kept at 1100 units per 1 cc. in the present series. In addition to these preparations, tube cultures were used in the following manner: Segments of 7 mm. glass tubing were cut to a length

---

* From the Department of Pathology of St. Joseph's Hospital, Houston, Texas. This investigation was supported by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council. Presented at the Eighth Annual Cancer Symposium, April, 1954, at the M. D. Anderson Hospital, Houston.

of approximately 6.5 cm. Five or six explants were attached to a 4 x 50 mm. coverslip in the manner previously described, using embryonic extract and chicken plasma for the clot. The coverslip was then inserted into the tube in such a way that it would lie within the curvature of the tube, the explants facing the glass wall. 0.15 to 0.2 cc. of human placental serum were injected between the coverslip and the tube wall, and the tube was sealed on both ends with rubber stoppers. The final penicillin concentration was 600 units per 1 cc. The coverslips in these preparations adhered to the tube walls and the tubes could be rotated without spilling the supernatant. They were placed in the incubator lying flat on one side, and were checked daily for out-growth under the low power lens of the microscope.

The preparation of the neotetrazolium-succinate mixture was identical with the previously described procedure, as was the incubation of the cultures in this solution. The following additional staining methods were used: 1. Brilliant Cresyl Blue in 1.0 per cent aqueous solution, applied for 30 minutes as a supravital stain. 2. Brilliant Cresyl Blue, as described under No. 1, applied as a counterstain following exposure of the culture to the neotetrazolium-succinate mixture. 3. Sudan Black B, used as a saturated solution in 70 per cent alcohol for 2 minutes. 4. Janus Green B in a .002 per cent solution (2 drops of a saturated solution of JGB in absolute alcohol diluted in 10 cc. Gey's solution, applied supravitally for a period of 5 minutes.)

For all staining procedures the coverslips were removed from the original preparations, briefly washed in Gey's solution, and then placed in the different stains as outlined above. After completion of the individual procedures the coverslips were washed in distilled water and mounted in glycerin.

Table 1 indicates the number of different staining procedures employed in the benign and malignant series of specimens.

Results

The results obtained after exposure of the out-growths to the neotetrazolium-succinate mixture are listed in tables 2 and 3. In conformance with the previously published method of interpretation the results were graded according to the amount

of colored matter noted at the end of the incubation in the mixture and were further qualified by the presence or absence of crystals. In general, formazan deposition was most marked within the planted piece, but particularly heavy staining of the explant was not necessarily associated with a positive reaction in the cells of the outgrowth. Rapid *in vitro* growth was not in-

TABLE 1

Designation of the different staining procedures employed and number of malignant or benign specimens which were used, in the form of in vitro out-growths, for each method.

	Malignant	Benign
Neotetrazolium-succinate, supravital	24	34
Brilliant Cresyl Blue, following Neotetrazolium-succinate	7	18
Brilliant Cresyl Blue, supravital	6	23
Sudan Black B, supravital	5	20
Janus Green B, supravital	4	20

variably accompanied by pronounced formazan production and many large deposits could be found in the cells of some poorly growing cultures. Deposits of the droplet type were usually seen where the cultures showed evidence of degeneration, but were, in some instances, also found in the earliest out-growths. The effects of the other staining procedures are summarized in table 4.

Discussion. The individual staining methods as outlined above were employed mainly in order to evaluate the following possibilities which constitute arguments and difficulties peculiar to the study of the tetrazolium effect in tissue cultures rather than in slices of fresh tissues.

1. The possibility of secondary uptake of formazan deposits by the cells of the out-growth.
2. A dependence of the reaction on necrobiosis.
3. The accumulation of formazans in lipids.
4. A possible interrelation between tetrazolium reduction and the degree of activity of the individual out-growths.

The uptake of deposits by the growing cells might take place after these particles have been formed within the planted piece. This possibility, if proven to be true, would seriously interfere with the validity of the reaction as applied to tissue culture work. However, this argument appears remote in view of the associ-

ation of negative out-growths with strongly positive reactions in the explants. Furthermore, a definite difference can be demonstrated between the distribution of the formazan deposits and

TABLE 2

Results observed in out-growths of 24 malignant specimens treated with the neotetrazolium-succinate mixture. +: "one plus"; ++: "two plus"; —: negative; (c): "crystals present."

Final Classification of Specimens	Tissue Culture Number	Results
Carcinoma of cervix (HeLa)	362	++ (c)
Wilms' tumor of kidney	365	++ (c)
Carcinoma simplex, Grade III, of breast	375, 441	++ (c), ++ (c)
Hodgkin's disease of cervical lymph node	376	+ (c)
Hypernephroid carcinoma of kidney	385	++
Metastatic tumor in cervical lymph node	403	+ (c)
Squamous cell carcinoma, metastasis to lymph node, Grade III	415	++ (c)
Leiomyosarcoma of duodenum	424	++ (c)
Scirrhous carcinoma, Grade III, of breast	427	++ (c)
Basal cell carcinoma of face	428	+ (c)
Adenoacanthoma, metastatic to axillary lymph node	432	++ (c)
Primary carcinoma of liver	435	—
Carcinoma simplex, Grade II, of breast	438	++ (c)
Malignant melanoma metastatic to chest wall	442	++ (c)
Adenocarcinoma, Grade II, of uterus	445, 465	++ (c), ++ (c)
Ascitic fluid with fragments of adenocarcinoma of ovary	446	++ (c)
Omentum with metastatic carcinoma of ovary	447	++
Squamous cell carcinoma, Grade II, of hand	449	—
Intraductal carcinoma, Grade II, of breast	461	+ (c)
Adenocarcinoma, Grade III, of prostate	473	+ (c)
Pleural fluid with metastatic carcinoma of ovary	479	++ (c)
Undifferentiated carcinoma of lung	488	++ (c)

particles of supravitally applied brilliant cresyl blue. The latter are evenly distributed and are found in practically all the cells, while the former are irregular in their pattern of distribution and are found only in a certain number of the cells of the outgrowth. The fact that tetrazolium reduction is favored by necro-

TABLE 3

Results observed in out-growths of 34 benign specimens treated with the neotetrazolium-succinate mixture. See TABLE 2 *for explanation of symbols.*

Final Classification of Specimens	Tissue Culture Number	Results
Proliferative endometrium with hyperplasia	359, 360, 405, 421, 429, 444	++ (c), ++ (c), ++ (c), —, ++ (c), + (c)
Secretory endometrium	363, 412, 453, 468	++ (c), + (c), +, + (c)
Bronchial adenoma	370	—
Fibroadenoma of breast	371, 437	—, + (c)
Pleural fluid, no evidence of malignancy	373, 398	+ (c), —
Proliferative endometrium	378, 383, 411	++ (c), ++ (c), —
Adenomatous polyp of uterus	384, 419, 480	—, + (c), +
Microfollicular adenoma of thyroid	394, 402	+ (c), —
Embryonal adenoma of thyroid	425, 472	—, ++ (c)
Nodular goiter	433	—
Non-specific hyperplasia of hilar lymph node	434	—
Intraductal papilloma of breast	448	—
Fibroadenoma of secreting breast tissue	450	—
Macro-follicular adenoma of thyroid	414, 452	—, —
Foreign body granuloma of inguinal lymph node	454	—
Fibrocystic disease of breast	457	—
Endometrium, resting stage	464	—

biosis has been pointed out by Straus, Cheronis, and Straus (1948). In working with tissue cultures we can minimize this factor by examining the cultures every day and using the earliest outgrowths for staining with neotetrazolium. The presence of formazan deposits in the cells of such cultures may be considered evidence against necrobiosis being a prerequisite for a positive reaction. The tendency of the formazans to be dissolved in lipid

droplets may lead to the impression that the appearance of visible deposits might depend on the lipid contents of the cells examined. This particular argument is easier to disprove than some of the others, since it can be shown that the droplet type of deposit is likely to be due to colored matter dissolved in lipid, but that the crystalline type is independent of the presence of lipids. The droplets take a lighter stain than the crystals, being usually pink to red, rather than purple or black. The pattern obtained after staining with Sudan Black B is quite similar to the distribution of these droplets and this close resemblance supports the ex-

TABLE 4

Characteristic effect of the different staining procedures.

Stain	Effect
Neotetrazolium-succinate, supravital	a. Droplet deposits: light red or pink b. Crystalline deposits: purple or black, rod-shaped or granular
Brilliant Cresyl Blue, supravital	Many punctate deposits crowded within the cytoplasm, found in all cells of viable out-growths
Brilliant Cresyl Blue, following Neotetrazolium-succinate	Diffuse blue coloration of cytoplasm and nuclei; formazan deposits unchanged
Sudan Black B, supravital	Black droplets, irregularly distributed, not present in all out-growths
Janus Green B, supravital	Diffusely distributed minute deposits in all cells of viable out-growths, no crowding.

planation that the droplet deposits are caused by an accumulation of lipids. The crystals are granular or rod-shaped and their distribution differs from that of the material demonstrated by the application of Sudan Black B. These precipitates are, therefore, considered a more reliable indicator of tetrazolium reduction than the light staining droplets. The argument that tetrazolium reduction might be governed by the growth rate of the culture and by other factors connected with *in vitro* conditions rather than by the type of planted tissue is the most serious problem among the various difficulties encountered. If this view were confirmed it would be impossible to draw any conclusions about the growth potential of any given specimen from the degree of formazan formation in its out-growth. To some extent the argument can be refuted by the observation of negative results in rapidly growing cultures and of strongly positive reactions

in the early out-growths of some sluggishly growing specimens. The previously reported absence of crystalline deposits in the fibroblastic type of growth also speaks against an explanation of the reaction on the basis of *in vitro* conditions alone. Nevertheless, we are as yet unable to deny that factors generally favoring the development of tissue cultures have an accelerating and increasing effect on the outcome of the tetrazolium reduction. There are, in addition, circumstances which are known to improve the reaction, such as an increase in pH and reduction of oxygen contents. Such changes are not an expression of the growth rate of the tissue *in vitro*, but they are difficult to standardize in short-term tissue cultures.

The cells stained with Janus Green contained many fine particles scattered throughout the cytoplasm. These were somewhat similar, in their distribution, to the punctate formazan deposits but varied from them by their small size and also by the uniformity of their appearance in all the cells of the different cultures examined. The Janus Green effect is generally thought to depend on the presence of mitochondria. The possibility of a similar connection cannot be denied for the tetrazolium reduction but the assumption of an identical mechanism governing both reactions appears unlikely.

In the final review the results obtained justify a cautious statement that formazan deposition, at least in its crystalline dark staining form, is an indication of cellular function rather than of secondary uptake. Whether the kind of original tissue or the growth rate of the culture is responsible for the final effect can, at present, not be decided with certainty. The "++ (c)" type of result predominates in the malignant group, but there are also some instances of this type among the benign specimens. Analysis of the non-malignant group shows the strongly positive reactions confined mainly to endometrium in the proliferative phase. This is consistent with the commonly accepted opinion that fast growing tissues generally produce the most marked degree of reduction. An encouraging sign is seen in the fact that malignant specimens usually produce "+ + (c)" reactions even though their out-growths are sometimes slow in developing. These findings are considered sufficiently significant to warrant further investigations with the object of finding differences between malignant and benign or non-neoplastic tissues based on the tetra-

zolium reduction by the cells of their out-growths. The procedure, so far, is still in a rather crude form, and many refinements should be possible. The actual staining technique has recently been modified by the addition of calcium chloride and sodium bicarbonate (Rutenburg, Wolman, and Seligman, 1953) and we are, at present, using the method of Wachstein and Meisel (1954) for a new series of cases. We are also diluting the tetrazolium solution with varying amounts of serum from different sources in an effort to determine if certain sera may have an inhibiting or activating effect on the reaction. The use of sub-cultures represents an additional modification which should eliminate any possible influence of the planted piece on the result. Finally, the role of the light in accelerating the reaction and favoring formazan deposition is being studied by means of exposure to a known light source for varying periods of time.

Summary. Out-growths from specimens of twenty-four (24) malignant tumors and thirty-four (34) benign lesions or normal tissues were incubated with a neotetrazolium-succinate mixture and the results recorded according to the amount and character of formazan deposition. In addition, several supra-vital staining procedures were applied and their results compared with the effect of tetrazolium reduction. The presence of formazan crystals in the cells of the out-growths is probably due to their formation in these cells rather than to secondary uptake and it is independent of the lipid contents of the cultures. It has, so far, not been possible to disprove the possibility of a connection between the degree of formazan deposition and conditions governing *in vitro* growth, but some of the findings suggest that the intensity of the reaction depends, instead, on the type of tissue used for the culture. Modifications and additional procedures are outlined which might help to overcome this problem and to refine the reaction so that it can perhaps be used in differentiating between benign lesions and malignant tumors.

Acknowledgments

The author is again indebted to Dr. C. M. Pomerat of The University of Texas, Medical Branch for advice and guidance as well as for the use of a HeLa subculture. Thanks are also due Mrs. Jacqueline Carlos, M. T., and Mrs. Martha Hunt, M. T., for their technical assistance.

REFERENCES

Marcuse, P. M.: Application of Neotetrazolium to Out-growths of Human Neoplasms in Tissue Culture. *Texas Rep. on Biol. and Med.*, 12: 126–137, 1954.

Rutenburg, A. M., M. Wolman, and A. M. Seligman: Comparative Distribution of Succinic Dehydrogenase in Six Mammals and Modification in the Histochemical Technic. *J. Histo. and Cytochem.*, 1: 66–81, 1953.

Straus, F. H., N. D. Cheronis, and E. Straus: Demonstration of Reducing Enzyme Systems in Neoplasms and Living Mammalian Tissues by Triphenyltetrazolium Chloride. *Science*, 108: 113–115, 1948.

Wachstein, M., and E. Meisel: Influence of Experimental Renal Damage on Histochemically Demonstrable Succinic Dehydrogenase Activity in the Rat. *Am. J. Path.*, 30: 147–165, 1954.

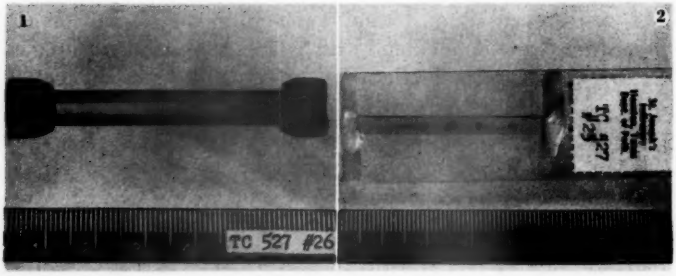
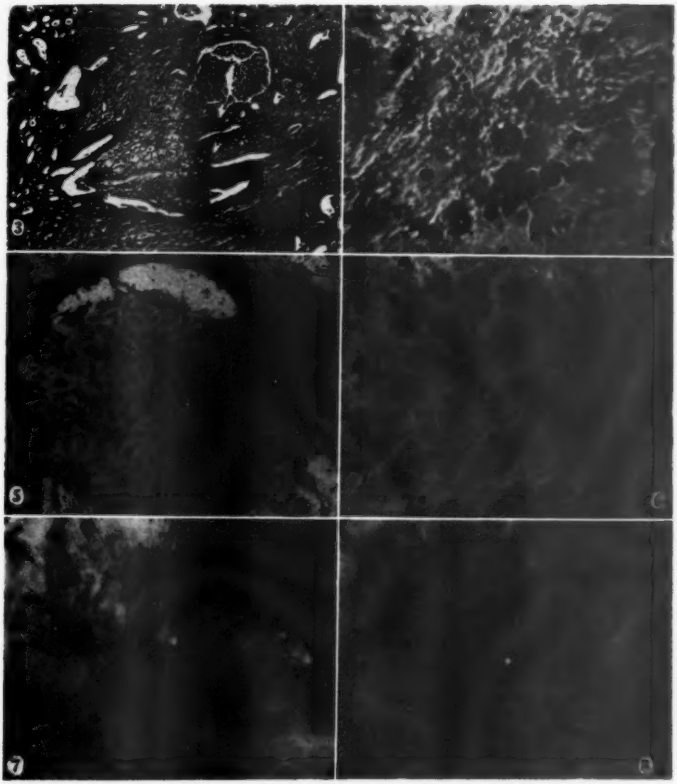


FIG. 1. Culture tube as used for the study of short-term cultures. The coverslip, bearing the planted pieces, is held in place through the presence of the liquid phase within the narrow upper space produced by the convexity of the tube wall.

FIG. 2. Final preparation with the coverslip mounted on a slide. The explants are stained dark red or purple after incubation in the neotetrazolium-succinate mixture.



FIGS. 3 to 11. T. C. 432. Adeno-acanthoma, metastatic to lymph node. These illustrations represent the complete work-up of the specimen, including histological section and the various stains of the out-growths.

FIG. 3. Histological section, demonstrating squamous elements as well as the adenomatous components of the tumor (Hematoxylin and eosin, X 100).

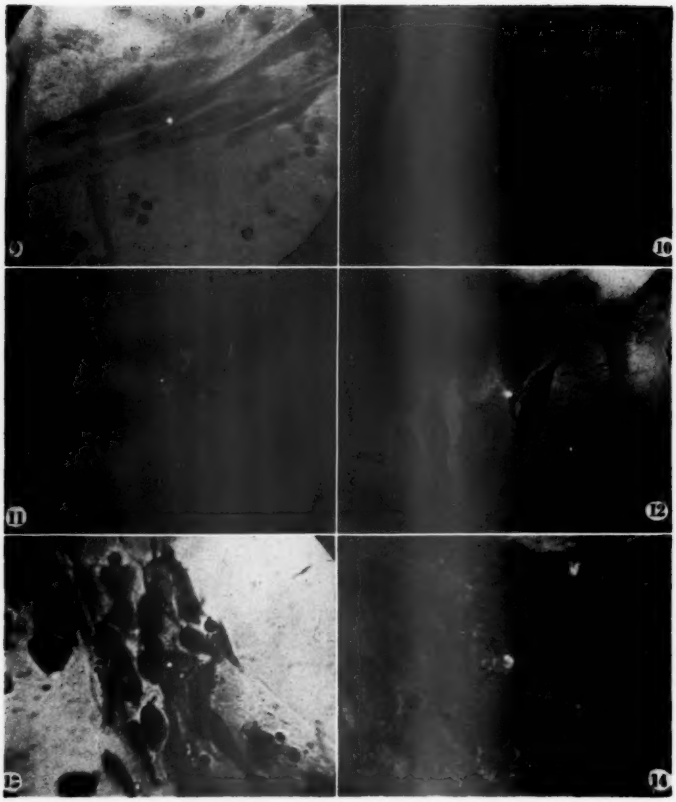
FIG. 4. Frozen section made from a block that had been incubated in the neotetrazolium-succinate mixture immediately after removal of the specimen. Of note are the heavy formazan deposits which have obscured the cytological details (X 940).

FIG. 5. Three-day-old out-growth composed of epithelial sheets (Jacobson's stain, X 430).

FIG. 6. Higher magnification of figure 5 shows cytological details of the culture. Most of the cells are cylindric epithelial in type (Jacobson's stain, X 940).

FIG. 7. Three-day-old culture, incubated in the neotetrazolium-succinate mixture and counterstained with Brilliant Cresyl Blue. The formazan deposits are plainly visible, even at this relatively low magnification (X 430).

FIG. 8. Higher magnification of figure 7. The distribution of the deposits is shown in relation to the cell borders and the nuclei (X 940).



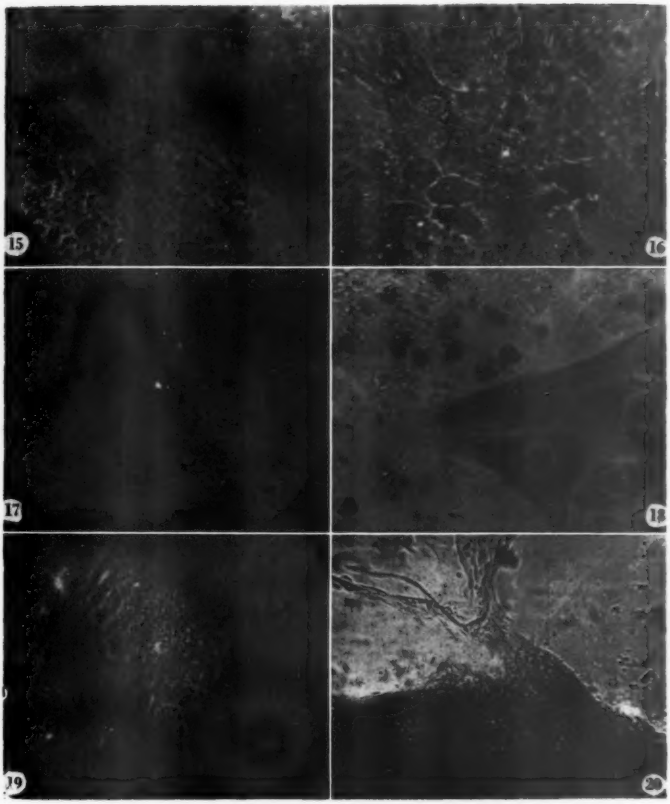
FIGS. 9, 10, 11. Effect of supravital staining with Brilliant Cresyl Blue (Fig. 9), Sudan Black B (Fig. 10), and Janus Green B (Fig. 11). Note the difference in the effect as compared with figure 8 (X 940).

FIGS. 12 TO 14. Endometrium, proliferative stage, in culture for 5 days, T. C. 421.

FIG. 12. Negative result of neotetrazolium application (X 940).

FIG. 13. Strongly positive effect of supravital Brilliant Cresyl Blue stain (X 940).

FIG. 14. Sudan Black B staining reveals fat droplets in some of the cells (X 940).



FIGS. 15 AND 16. Endometrium, secretory stage, 5 days in culture. T. C. 430. Compare the numerous lipid droplets with the scanty formazan deposits.

FIG. 15. Lipid droplets stained with Sudan Black B (X 460).

FIG. 16. Formazan deposits, "+ (c)" result (X 940).

FIGS. 17 TO 19. Microfollicular adenoma of thyroid, 5 days in culture. T. C. 394. Of note is the difference in the distribution of the stained particles, particularly the crowding in figure 18 as compared with the patchy formazan deposits in figure 17.

FIG. 17. Formazan deposition of "+ (c)" type (X 970).

FIG. 18. Brilliant Cresyl Blue, supravital (X 970).

FIG. 19. Janus Green B, supravital (X 970).

FIG. 20. Adenomatous polyp of uterus, in culture for 3 days. T. C. 480. (Neotetrazolium-succinate, X 220). Note that the planted piece has strongly reacted while the out-growth has only yielded a "+" result.

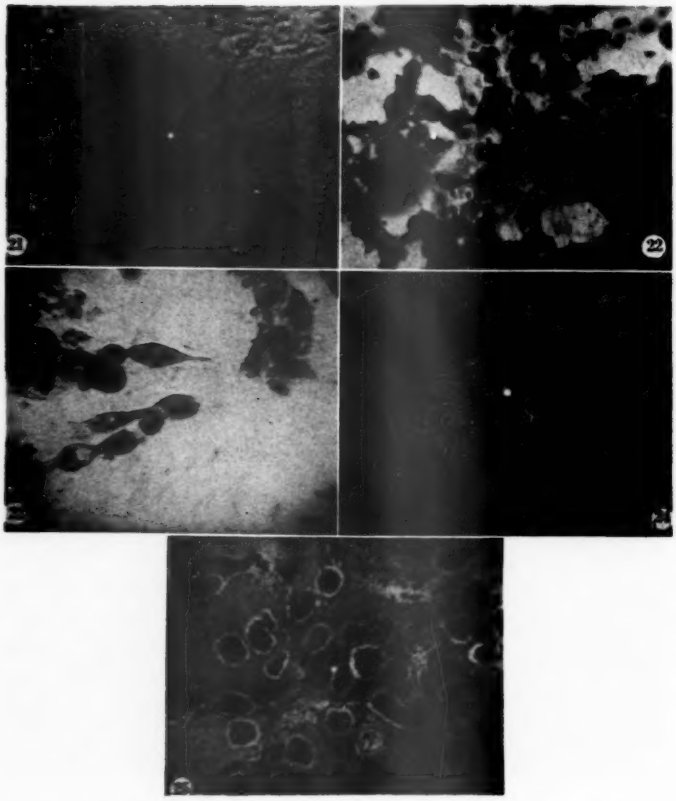


FIG. 21. Ascitic fluid with cells from adenocarcinoma. T. C. 446. (Neotetrazolium-succinate, X 940). This material had been in culture for only one day. There was scanty growth of only occasional cells but a "++ (c)" result was obtained.

FIGS. 22 AND 23. Fibrocystic disease of breast. T. C. 457.

FIG. 22. Four-day culture, stained by Jacobson's method to demonstrate satisfactory sheet growth (X 645).

FIG. 23. Four-day culture, incubated with neotetrazolium-succinate and yielding a negative result (X 940).

FIGS. 24 AND 25. Subcultures of cervical carcinoma (HeLa). T. C. 362.

FIG. 24. Typical "++ (c)" effect of neotetrazolium (X 940).

FIG. 25. Supravital stain with Brilliant Cresyl Blue (X 940).

CYTOLOGIC AND BIOCHEMICAL STUDIES ON THE GRANULOCYTES IN EARLY LEUKEMIA AMONG ATOMIC BOMB SURVIVORS*

WILLIAM C. MOLONEY† AND ROBERT D. LANGE‡

During the medical surveys of adult atomic bomb survivors carried out in the Atomic Bomb Casualty Commission Clinic in Hiroshima, Japan, nine cases of leukemia were encountered (Lange, *et al.*) In addition, four individuals were found with changes in the peripheral blood consisting of slight leukocytosis, presence of a small percentage of immature myeloid cells and increased percentage of basophils (Moloney, *et al.*). On further investigation of these patients, it was found that absolute basophil counts were greatly elevated and that the separated granulocytes were very low in alkaline phosphatase activity. Observations over periods of 5 to 37 months have indicated that these findings furnish highly significant evidence of early myelogenous leukemia and an account of these cases is presented in this report.

Methods and Materials. These four cases were discovered among adult survivors exposed under 1500 meters from the hypocenter (or ground zero). In the medical surveys a detailed history, including radiation history and physical examination with laboratory studies (complete blood, urine, serology, stool, and X-ray of the lungs) were carried out. Normal individuals were re-examined on an annual basis; those with abnormal findings were rescheduled for further investigation. In cases suspected to be early myelogenous leukemia, repeat blood and bone marrow studies were performed. Absolute basophil counts were done by counting basophils in 4000 leukocytes in a Wright-Giemsa

---

* From the Laboratories of the Atomic Bomb Casualty Commission, Hiroshima, Japan. This work was sponsored by the Atomic Bomb Casualty Commission, Field Agency of the National Academy of Sciences, National Research Council, with funds supplied by the United States Atomic Energy Commission. Presented at the Eighth Annual Cancer Symposium, April, 1954, at the M. D. Anderson Hospital, Houston.

† Medical Director, Atomic Bomb Casualty Commission, on leave of absence from the Tufts Medical School, Boston, Massachusetts.

‡ Internist, Atomic Bomb Casualty Commission, on leave of absence from the Washington University School of Medicine, St. Louis, Missouri.

stained peripheral blood smear and the number per cu. mm. calculated from the white blood count (Moore and James, 1953). Alkaline and acid phosphatase activity and glycogen content of separated leukocytes were determined using the techniques employed by Valentine and Beck (1951).

Comment

General Data and Radiation History. As noted in table 1, all four cases were within range of intense irradiation; however, Case 3 (K.S.), although exposed at 650 meters, was in a concrete building and did not develop severe radiation symptoms. These patients were all seen on routine surveys and had no complaints or abnormal physical findings at the time of their initial visits. The course of the hematological picture and the biochemical data are presented in tables 2, 3, 4, and 5.

General Hematological Features. The initial findings in the peripheral blood which suggested the possibility of leukemia were slight leukocytosis with small numbers of immature myeloid cells and increased, often atypical, basophils. The leukocyte count was variable, but slight to marked leukocytosis was present in all cases, tending to rise gradually over a period of months. Platelets were increased sometimes to a tremendous degree (as in Case 3, K.S.). Anemia was not an outstanding feature of the picture, although changes in the red cells, such as anisocytosis with slight macrocytosis, polychromasia, and occasional nucleated red cells, were seen without reduction in the hemoglobin or red cells.

The leukocytes in the peripheral smear were mainly of the neutrophilic granulocyte group, and there was a consistent relative lymphopenia. In the early stages, band forms were only slightly increased, in contrast to the granulocytosis of infection. The presence of metamyelocytes and myelocytes, although in small numbers, was an outstanding feature of the peripheral blood picture, and the "hiatus" between the segmented neutrophils and immature myeloid cells was noteworthy.

Eosinophilia. In the hematological picture of early myelogenous leukemia, eosinophilia has been described. However, due to the prevalence of intestinal parasites, eosinophilia in the Japanese was of little value in the diagnosis of these cases.

TABLE 1

General and Radiation Data on Four Cases of Preclinical Myelogenous Leukemia Some of These Cases Showed Cataract

Case	Date of Birth	Sex	Exposure in Meters*	Epilation	Oropharyngeal Lesions	Purpura	Nausea-Vomiting	Diarrhea	Fever	Injuries	Burns	Date of First Visit	Date of First Hematological Evidence
No. 1, S.Y.	1/18/00	M	400	0	0	0	X	X	0	X	0	6/ 7/51	6/ 7/51
No. 2, K.M.	3/15/93	F	1390	0	0	X	0	0	0	X	X	12/20/51	12/20/51
No. 3, K.S.	8/ 7/02	M	650	0	0	0	0	0	X	X	0	12/12/50	4/17/52
No. 4, I.H.	12/11/15	M	1170	90%	X	X	X	X	X	0	0	8/26/53	8/26/53

* From hypocenter or ground zero.

TABLE 2

Case No. 1, S.Y., M.F. No. 220, 344. Male, Age 54. Exposed 400 Meters.
Hematological and Biochemical Data.

Date	Hb.	R.B.C.	W.B.C.	Platelets	Differential — Peripheral Blood	Absolute Basophils* Per Cu. Mm.
7/ 7/51	15.9	5.34	11,800		Seg. N., 58; Band N., 3; Metamyelo. N., 6; Myelo. N., 1; Eosin., 4; Baso., 5; Lymph., 20; Mono., 3.	286
7/31/51	15.1	4.99	10,950	141,000	Seg. N., 50; Band N., 2; Metamyelo. N., 3; Myelo. N., 2; Promyelo., 1; Eosin., 2; Baso., 5; Lymph., 28; Mono., 7.	397
7/ 8/52	14.7	4.80	12,050		Seg. N., 55; Band N., 3; Metamyelo. N., 6; Myelo. N., 1; Eosin., 4; Baso., 5; Lymph., 20; Mono., 3.	. . .
6/ 2/53	15	5.08	16,375		Seg. N., 67; Metamyelo., N., 3; Myelo. N., 4; Eosin., 1; Baso., 7; Lymph., 14; Mono., 4.	819
6/16/53			14,250		Seg. N., 51; Band N., 1; Metamyelo. N., 11; Myelo. N., 4; Eosin., 3; Baso., 7; Metamyelo. Baso., 1; Lymph., 17; Mono., 6.	606
					Alk. Phos., 2.0; Ac. Phos., 17.6; Glucose, 8.4.	
Differential—Bone Marrow: Seg. N., 24.5; Band N., 23.5; Metamyelo. N., 29.5; Myelo. N., 17; Promyel., 1.5; Myelobl., 0.5; Eosin., 0.5; Band E., 2; Metamyelo. E., 1.5.						
8/25/53	14.4	4.89	14,875		Seg. N., 57; Band N., 1.5; Metamyelo. N., 4; Myelo. N., 2.5; Promyel., 0.5; Eosin., 2; Band E., 1; Baso., 8.5; Metamyelo. Baso., 0.5; Lymph., 16; Mono. 6.5. R. B. C. platelets normal.	788
					Alk. Phos., 1.8; Ac. Phos., 12.5; Glycogen, 3.1.	
Differential—Bone Marrow: Seg. N., 19; Band N., 24.5; Metamyelo. N., 24; Myelo. N., 25; Promyel., 3.5; Myelobl., 0.5; Eosin., 1; Metamyelo. E., 2; Baso., 1. R. B. C. activity reduced.						
11/ 3/53	13.3	5.01	22,975	741,480	Seg. N., 67; Band N., 3; Metamyelo. N., 7; Myelo. N., 1; Eosin., 2; Baso., 5; Lymph., 12; Mono., 3. R.B.C. occasionally polychromatophilic, platelets plentiful.	840
					Alk. Phos., 4.7; Ac. Phos., 29.7; Glycogen, 5.9.	
Differential—Bone Marrow: Seg. N., 32; Band N., 24; Metamyelo. N., 24; Myelo. N., 8.8; Promyelo., 8.0; Eosin., 0.8; Band E., 0.4; Baso., 0.4; Lymph., 1.2; Mono., 0.4.						
12/ 8/53	14.2	4.87	22,300	1,139,580	Seg. N., 68.8; Band N., 3.4; Metamyelo. N., 4.2; Myelo. N., 0.4; Seg. E., 2.6; Band E., 02; Baso., 3.8; Lymph., 9; Mono., 7.6 R.B.C. normal.	669
					Alk. Phos., 28†; Ac. Phos., 46.6†; Glycogen, 6.0‡	

* Dry method — 4,000 cell count.
† Expressed as mg : P liberated per hour per 10^{10} leucocytes.
‡ (Glucose or glycogen) as mg.

TABLE 3

Case No. 2, K.M., M.F. No. 256,175. Female, Age 61. Exposed 1390 Meters. Hematological and Biochemical Data.

Date	Hb.	R.B.C.	W.B.C.	Platelets	Differential — Peripheral Blood	Absolute Basophils* Per Cu. Mm.
12/20/51	12.3	3.99	10,550	214,000	Seg. N. 62; Band N., 5; Eosin., 3; Baso., 4; Lymph., 22; Mono., 4.	617
2/ 5/52			11,100		Seg. N., 61; Band N., 5; Metamyelo. N., 1; Eosin., 1; Baso., 7; Lymph., 21; Mono., 4.	
4/ 1/52	10.7	3.93	12,100	573,080	Seg. N., 55; Band N., 8; Eosin., 1; Baso., 8: Lymph., 19; Mono., 7; Unclass., 2.	662
Differential—Bone Marrow: Seg. N. 20; Band N., 28.5; Metamyelo. N., 25.5; Myelo. N., 16; Promyel., 1; Myelobl., 1; Eosin., 2.5; Band E., 0.5; Lymph., 3; Plasma cell, 2.						
3/11/53	11.1	4.02	7,325		Seg. N. 57.5; Band N., 4.5; Eosin., 2.5; Metamyelo. E., 0.5; Baso., 3; Lymph., 24; Mono., 4.5; Unclass., 4.	470
4/23/53	11.5	3.94	11,300		Seg. N 42; Band N., 2; Eosin., 9; Baso., 8; ? Myelo, 2; Lymph., 32; Mono., 8.	1,039
Alk. Phos., 0.9;† Ac. Phos., 12.0;† Glycogen, 48.4.‡						
Differential—Bone Marrow: Seg. N., 14; Band N., 13; Metamyelo. N., 25.5; Myelo. N., 27.5; Promyelo., 2; Myelobl., 1.5; Eosin., 4.5; Myelo. E., 4.5; Lymph., 5.5; Retic. cell, 1; Plasma cell, 1.						
7/ 9/53	10.8	3.41	10,325		Seg. N., 53; Band N., 3; Metamyelo. N., 6; Eosin., 5; Matamyelo. Baso., 2; Baso., 9; Mono., 1; Lymph., 21.	730
9/22/53	12.3	4.18	8,425	342,760	Seg. N., 63; Metamyelo. N., 0.5; Eosin., 1.5; Baso., 9.5; Lymph., 21.5; Mono., 3. R.B.C. and platelets normal.	619
Alk. Phos., 1.17†; Ac. Phos., 30.4†; Glycogen, 6.40‡.						

* Dry method — 4,000 cell count.
† Expressed as mg: P liberated per hour per 10^{10} leucocytes.
‡ (Glucose or glycogen) as mg.

TABLE 4

Case No. 3, K.S., M.F. No. 284,913. Male, Age 52. Exposed 650 Meters. Hematological and Biochemical Data.

Date	Hb.	R.B.C.	W.B.C.	Platelets	Differential — Peripheral Blood	Absolute Basophils* Per Cu. Mm.
12/12/50	16.7	5.39	12,050	150,000	Seg. N., 64; Eosin., 5; Baso., 2; Mono., 4; Lymph., 24. R.B.C. normal; platelets increased, atypical.	238
4/17/52	14.6	4.97	12,550	576,520	Seg. N., 76; Band N., 1.5; Myelo. N., 0.5; Eosin., 4; Baso., 7.5; Lymph., 10; Mono., 1. R.B.C. slightly aniso. and poikilo; platelets + + +.	696
6/18/53	13.2	4.42	14,250		Seg. N., 54; Band N., 6; Metamyelo. N., 2; Seg. Baso., 1; Band B., 2; Metamyelo. B., 15; Myelo. B., 2; Lymph., 15; Mono., 2; Retic. Cell 1. R.B.C. and platelets normal.	1,899
6/23/53	12.1	4.01	17,675		Seg. N., 56; Band N., 5; Metamyelo. N., 2; Myelo. N., 1; Promyelo., 1; Eosin., 4; Baso., 15; Band B., 2; Metamyelo. Baso., 3; Lymph., 11. R.B.C. show aniso. and poikilo. Platelets + + +, masses.	2,875
					Alk. Phos., 12.2; Ac. Phos., 44.9; Glucose, 38.5.	
12/15/53	9.24	3.08	39,150	6,622,000	Seg. N., 36.2; Band N., 8.4; Metamyelo. N., 6.0; Myelo. N., 1.2; Promyel., 0.8; Seg. E., 5.6; Band E., 1.6; Metamyelo. E., 0.6; Myelo. E., 0.4; Baso., 24.6; Lymph., 11.2; Mono., 2.4; Plasma cell, 0.2; Blast, 0.8. Anisocytosis, +; platelets markedly abundant.	8,221
					Alk. Phos., 1.7†; Ac. Phos., 18.1†; Glycogan, 13.2‡.	
1/18/54	9.3	3.50	42,500	2,094,500 Retic. 1.8	Seg. N., 27.6; Band N., 18; Metamyelo. N., 15.4; Myelo. N., 12; Promyel., 2.4; Eosin., 3.2; Band E., 1.0; Metamyelo. E., 0.2; Myelo. E., 0.2; Baso., 15.4; Lymph., 0.6; Mono., 1.4; Blast, 2.6. N.R.B.C., 3/500 Wbc; megakaryocytes on smear.	7,905

* Dry method — 4,000 cell count.
† Expressed as mg : P liberated per hour per 10^{10} leucocytes.
‡ (Glucose or glycogen) as mg.

TABLE 5

Case No. 4, I.H., M.F. No. 256,655. Male, Age 39. Exposed 1170 Meters. Hematological and Biochemical Data.

Date	Hb.	R.B.C.	W.B.C.	Platelets	Differential — Peripheral Blood	Absolute Basophils* Per Cu. Mm.
8/26/53	12	4.39	13,300		Seg. N., 53; Band N., 2; Metamyelo. N., 4; Myelo. N., 7; Promyelo., 1; Seg. E., 8; Baso., 4; Lymph., 13; Mono., 8. R.B.C. show polychromatophilia and stippling; platelets plentiful.	665
					Alk. Phos., 8.5; Ac. Phos., 14.9; Glycogen, 5.9.	
Differential—Bone Marrow: Seg. N., 17; Band; N., 38; Metamyelo. N., 25; Myelo. N., 7; Promyel., 2; Eosin., 2; Band E., 3; Metamyelo. E., 6, Red cell activity reduced. Mitoses increased. Megakaryocytes normal.						
8/28/53	13	4.67	19,875	434,310	Seg. N., 50; Band N., 3; Metamyelo. N., 11; Myelo. N., 2; Seg. E., 12; Band E., 1; Baso., 3; Lymph., 13; Mono., 2. R.B.C. occasionally polychromatophilic; platelets normal.	914
					Alk. Phos., 1.2; Ac. Phos., 19.3; Glycogen, 3.7.	
11/21/53	12.3	4.61	15,417	691,500	Seg. N., 48.4; Band N., 9.4; Metamyelo. N., 10.2; Myelo. N., 2.0; Promyel., 0.2; Seg. E., 8.2; Band E., 0.8; Baso., 4.6; Lymph., 10.2; Mono., 5.4; Retic. Cell, 0.2; Plasma cell, 0.4.	532
					Alk. Phos., 2.4; Ac. Phos., 17.8; Glycogen, 2.1.	
12/14/53	12.3	4.22	15,725	210,040	Seg. N., 62.6; Band N., 4.6; Metamyelo. N., 7.6; Seg. E., 4.4; Band E., 0.4; Baso., 6.2; Lymph., 10.0; Mono., 4.2. Platelets abundant. Alk. Phos. 3.8†; Ac. Phos., 25.3†; Glycogen, 2.6‡.	676

* Dry method — 4,000 cell count.
† Expressed as mg : P liberated per hour per 10^{10} leucocytes.
‡ (Glucose or glycogen) as mg.

Basophilocytosis. A striking feature of these four cases was the marked increase in basophils in the peripheral blood, even in the earliest stages of the disease. Basophilocytosis was especially significant since there are few diseases, except chronic myelogenous leukemia, in which a marked increase in basophils occurs. It has been pointed out that routine peripheral blood differential counts often give erroneous results; nevertheless in routine hematological studies on 2,162 Japanese adults the basophils averaged 0.6 per cent and basophil counts over 2 per cent were rare. Using the more accurate method based on counting basophils in 4,000 leukocytes on a stained coverslip smear (Wright-Giemsa), absolute basophil counts per cu. mm. were determined on 60 normal Japanese adults. Of this group, 52 were found to have less than 50 and only 8 between 50 and 99 basophils per cu. mm. The average for the group was 28.1 per cu. mm. and the range 5 to 67 per cu. mm. In a total of 80 observations on 11 well established and 6 early cases of chronic myelogenous leukemia studied at the ABCC Clinic greatly elevated absolute basophil counts were consistently demonstrated. Except for the early myelogenous leukemia cases, no instances of abnormal elevation of the basophil counts were encountered in the 4,200 exposed and unexposed Japanese adults studied in Hiroshima. However, among cases referred for diagnostic study, two unexposed patients with leukemoid blood pictures and polycythemia vera were found to have an increased number of basophils in the peripheral blood (see FIG. 1).

Bone Marrow Studies. In the early phase of chronic myelogenous leukemia, bone marrow aspiration carried out in three of the four cases was not helpful in diagnosis. While myeloid immaturity was present, the picture was not pathognomonic and might have been attributed to infection, "leukemoid reaction" or other causes. Case 3, K.S., with a most interesting blood picture of 24 per cent basophils, refused to permit bone marrow aspirations.

Biochemical Studies on Separated Leukocytes. In the past few years there has been increasing interest in biochemical and metabolic studies on isolated human leukocytes. Valentine and Beck (1951) found that in normal subjects alkaline phosphatase activity expressed in mg. of P liberated per hour by 10^{10} leukocytes ranged from 13.4 to 58.0 with a mean of 25.8. In subjects

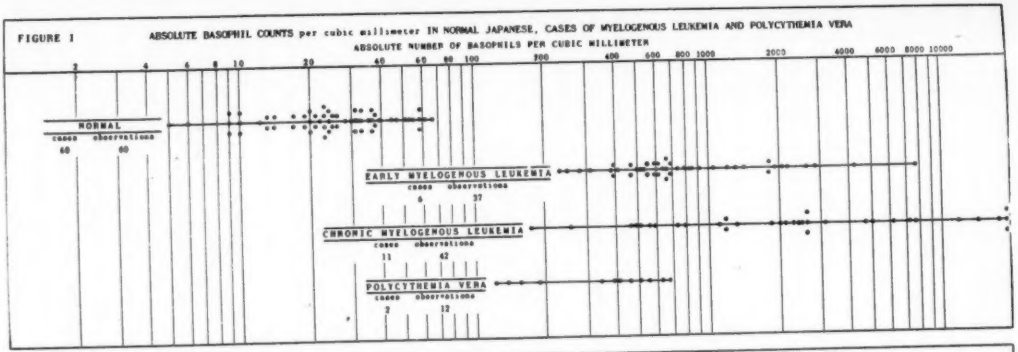


FIGURE I
ABSOLUTE BASOPHIL COUNTS per cubic millimeter IN NORMAL JAPANESE, CASES OF MYELOGENOUS LEUKEMIA AND POLYCYTHEMIA VERA
ABSOLUTE NUMBER OF BASOPHILS PER CUBIC MILLIMETER
2 4 6 8 10 20 40 60 80 100 200 400 600 800 1000 2000 4000 6000 8000 10000
NORMAL
cases observations
60 60
EARLY MYELOGENOUS LEUKEMIA
cases observations
6 37
CHRONIC MYELOGENOUS LEUKEMIA
cases observations
11 42
POLYCYTHEMIA VERA
cases observations
2 12

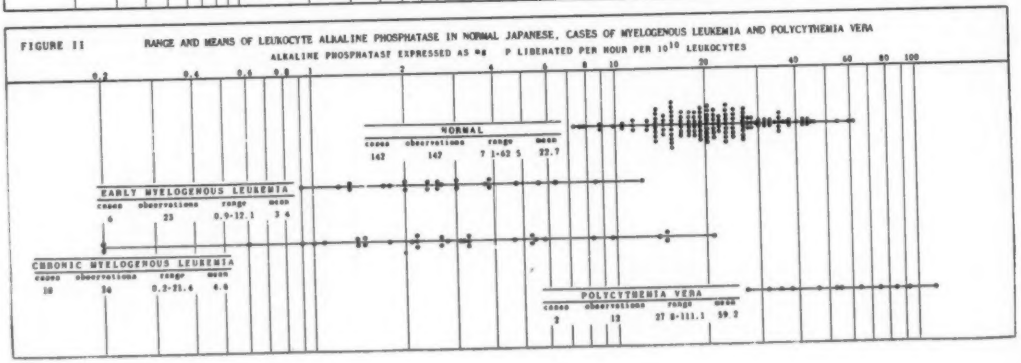


FIGURE II
RANGE AND MEANS OF LEUKOCYTE ALKALINE PHOSPHATASE IN NORMAL JAPANESE, CASES OF MYELOGENOUS LEUKEMIA AND POLYCYTHEMIA VERA
ALKALINE PHOSPHATASE EXPRESSED AS mg P LIBERATED PER HOUR PER 10^{10} LEUKOCYTES
0.2 0.4 0.6 0.8 1 2 4 6 8 10 20 40 60 80 100
NORMAL
cases observations range mean
142 147 7.1-62.5 22.7
EARLY MYELOGENOUS LEUKEMIA
cases observations range mean
6 23 0.9-12.1 3.4
CHRONIC MYELOGENOUS LEUKEMIA
cases observations range mean
10 34 0.3-21.4 4.6
POLYCYTHEMIA VERA
cases observations range mean
2 13 27.8-111.1 59.2

with leukocytosis due to infection, the range was 35.0 to 278.5, with a mean of 119.1, and they found that cases of polycythemia vera with leukocytosis had a similar pattern. In contrast, in 14 cases of chronic myelogenous leukemia, with one exception, the alkaline phosphatase values were very low, ranging from 0.0 to 14.4 with a mean of 4.0.

In the present investigation of leukemia occurring in atomic bomb survivors, biochemical studies of alkaline phosphatase, acid phosphatase, and glycogen have been carried out on separated leukocytes. Acid phosphatase activity proved to be variable and inconsistent; glycogen content of leukemia granulocytes was, in general, low. Due to the lack of technical facilities, histamine determinations could not be carried out.

As shown in figure II, the results of the studies on alkaline phosphatase activity in normal and leukemia leukocytes closely paralleled the findings of Valentine and Beck (1951). Repeated determinations of alkaline phosphatase activity demonstrated that in these four cases normal-looking granulocytes were as low in alkaline phosphatase activity as the granulocytes in well established cases of myelogenous leukemia.

Discussion

In this report the cytological aspects and the biochemical parameters of separated leukocytes in four cases of preclinical myelogenous leukemia are described. Slight leukocytosis with peripheral blood findings featuring small numbers of immature myeloid cells and an increased percentage of basophils were the earliest hematological clues. Absolute basophil counts were found to be markedly elevated and alkaline phosphatase activity of the separated leukocytes was consistently low, even in the early stages of the disease. These findings confirm the biochemical observations of Valentine (1951) and lend support to the concept that enzyme and biochemical changes may occur in leukemic granulocytes many months before cytological immaturity is evident. It is also apparent that the presence in the peripheral blood of even small numbers of metamyelocytes and myelocytes, with an increased absolute basophilocytosis, augmented by the findings of abnormally low alkaline phosphatase activity in the separated granulocytes, constitutes strong presumptive evidence of

early myelogenous leukemia in individuals exposed to ionizing irradiation.

Summary

Hematological and biochemical findings in separated leukocytes of four cases of preclinical myelogenous leukemia developing in atomic bomb survivors are described.

In the peripheral blood of individuals exposed to ionizing irradiation, the presence of even a small number of immature myeloid cells, slight leukocytosis, increased absolute basophil counts and low alkaline phosphatase activity of the separated leukocytes is highly suggestive evidence of early myelogenous leukemia.

Low alkaline phosphatase values in separated granulocytes in the early stages of myelogenous leukemia demonstrate that biochemical changes may precede cytologic evidence of immaturity by many months.

Acknowledgments

The authors gratefully acknowledge the technical assistance of Dr. Mizokoshi and Miss K. Tsuchitori in the biochemical and basophil studies carried out in these patients and of Miss Irene Kabat in the preparation of the manuscript.

REFERENCES

Lange, R. D., W. C. Moloney, and T. Yamawaki: Leukemia in Atomic Bomb Survivors. I. General Observations. *Blood.*, 9: 574, 1954.

Moloney, W. C., and R. D. Lange: Leukemia in Atomic Bomb Survivors. II. Observations on Early Phases of Leukemia. *Blood.*, 9: 663, 1954.

Moore, J. E., and G. W. James: A Simple Direct Method for Absolute Basophil Leukocyte Count. *Proc. Soc. Exp. Biol. and Med.*, 82: 601, 1953.

Valentine, W. N., and W. S. Beck: Biochemical Studies on Leukocytes. I. Phosphatase Activity in Health, Leukocytosis and Myelocytic Leukemia. *J. Lab. and Clin. Med.*, 38: 39–55, 1951.

Valentine, W. N.: Quantitative Biochemical Studies on Leukocytes in Man: A Review. *Blood*, 6: 845–854, 1951.

THE CLASSIFICATION AND DIAGNOSIS OF HUMAN TUMORS BY TISSUE CULTURE METHODS*

MARGARET R. MURRAY AND ARTHUR PURDY STOUT

One of the more difficult problems facing the practising oncologist is to recognize the nature of some of the less common tumors. Confusion may arise in this area either through the inadequacy of classical clinical and pathological methods to furnish a *differential* diagnosis where the alternatives are generally understood, or through the lack of a histogenetic concept with which to align a new unknown. In either situation, examination of the living, functioning neoplastic cell may contribute importantly, and sometimes crucially, to a solution of the problem.

Rationale. The living cell has a repertory of *modulations*, or reversible changes of form and function in response to environment (Bloom, 1937; Weiss, 1939), which may be used to advantage in tracing its tissue origin. Normal adult tissues,—for example skeletal muscle—, may assume *in vitro* a variety of forms not ordinarily encountered *in vivo*, but observable in the embryonic, regenerative or neoplastic state; conversely, neoplastic cells may be identified by their resemblance to such atypical normal cells (Stout, 1953; Timofeevskii, 1946–47). Though the range of modulations characterizing one tissue type may overlap that of another, there often exists a syndrome of morphological and functional features which are histogenetically distinctive. Intrinsic tissue characteristics may be emphasized by the changed environment of the culture, where the migrating cells are freed from the compression to which they are subject *in vivo*, and where the cell can always be seen in its entirety.

Uses. Not all tissue types are equally favorable for this kind of observation. Epithelial tissues unless specially handled tend to revert *in vitro* to a relatively undifferentiated squamous type of growth and to lose their topographical relationships, which are of diagnostic value in the classic pathological sections. In the latter

* From the College of Physicians and Surgeons and Francis Delafield Hospital, New York. This investigation was supported in part by research grant C-1752 from the National Cancer Institute, Public Health Service, and in part by the Margaret Fisher Fund. Presented at the Eighth Annual Cancer Symposium, April, 1954, at the M. D. Anderson Hospital, Houston.

also the presence of mitoses and their number per field are of diagnostic importance; the significance of such observations is reduced in culture, since mitosis is common in cultured normal adult cells.

In nervous tissues, however, distinguishing characteristics are generally intensified by cultivation *in vitro*. The most obvious advantage of the roomier culture environment is to allow the projection and expansion of cell processes, which, in the case of the neuron, are immediately recognizable. Supporting cells also, such as the Schwann cell, oligodendroblast and astroblast, assume much more distinctive forms *in vitro* than can be seen in sections, either normal or neoplastic.

For our purpose the mesoblastic tissues in general occupy an intermediate position between those of nervous derivation and the covering and lining epithelia. For most mesoblastic tumors tissue culture is best used in conjunction with clinical and routine pathological observations (Stout, 1953); it may provide a means of deciding among several alternatives left open by those methods,—for example, the choice between liposarcoma, rhabdomyosarcoma and fibrosarcoma or myxoma. A mesothelioma may be detected by this means (Stout and Murray, 1942), or distinctions made among lymphomas.

Earlier work. The idea of utilizing tissue culture to throw light on histogenetic relationships has occurred more or less independently to a number of investigators. Maximow (1924) was probably the first to realize it; from 1914 until his death he enlarged and modified his unitarian theory of blood and connective tissue origins mainly through observations on the living cell. Chlopin, a student and colleague of Maximow in Russia, was one of the first to perceive the possibilities implicit in the tissue culture method, for the study of the whole subject of histogenesis; and he exploited them ably in a series of papers beginning in 1922, dealing with various normal tissues derived from the three germ layers (Chlopin, 1932, 1933, 1937).

The idea of utilizing the tissue culture method for unravelling the histogenetic aspects of tumors seems to have originated in the Cushing Clinic with Kredel in 1928, at a time when recent advances in neurosurgery had made an expansion of diagnostic measures imperative. In 1933 Russell and Bland entered the

field, making a series of valuable contributions to our present day classification systems for neurogenous tumors. Time and space do not permit mention of the many other workers in the area of tumor classification. We shall attempt here only to illustrate some of the work that has been carried on in the Laboratory for Cell Physiology at the College of Physicians and Surgeons.

Method. Basically our method (like that of Russell and Bland) has been to compare the cultural characteristics of the tumor type in question with those of the normal tissues with which it might be thought to be related. Thus neurilemomas were compared *in vitro* with embryonic and adult Schwann cells and endo-perineurium.

A standard medium-composition is used throughout, consisting of human placental serum, chick embryo extract and plasma, and beef serum ultrafiltrate (Murray, 1951). Slight modifications are made in such matters as relative proportions of these ingredients, pH, frequency of feeding, etc., to accommodate the requirements of different tissue types, *e.g.*, epithelium vs. fibroblasts. For general purposes of observation and photography, cultures are made on double-coverslips using the Maximow-type depression slide. Stock cultures are often carried in Gey-type tubes, sometimes with Leighton-sponges. The culture period is short, however; from two weeks to at most two months. It has been found that if distinctive morphological characteristics are going to be revealed, they will appear well within those time-limits. It is essential that the cells should not be stimulated to rapid growth, since this favors genetic mutation and discourages specialization of form and function.

Observations such as we have made by these methods have already been extended into new areas by other culture techniques such as Leighton's (1951) sponge-method, and by organ culture in general (Fell, 1951); by time-lapse cinemicrographic records such as those by Canti, Bland and Russell, Lewis, Gey, and Pomerat; and by histochemical studies such as those of Marcuse. It is to be expected that important advances will take place in the near future through all these lines of effort.

Classification of Tumors

Neurilemoma. One of the earliest and clearest examples of the use of tissue culture in tumor classification is found in the specific

nerve sheath tumor, or neurilemoma. This has been called by many names since its original designation by Verocay in 1908 as "neurinoma"; among these are Schwannoma and perineural fibroblastoma. The essential controversy hinged on which element of the peripheral nervesheath this tumor was derived from,—the Schwannian component or the fibroblastic, a problem that was refractory to solution by standard histological methods.

Our mode of attack was to culture the normal peripheral nerve, in the expectation that differences could be observed between the deportment *in vitro* of the various components. If the normal Schwann cell could be distinguished from the endo-perineural cell in tissue culture (Plate I, Fig. 4), where it can be seen as a whole and does not depend for identification upon its relationship to the nerve fiber or bundle, then it should be possible by cultivating the neurilemoma, to determine whether this tumor is derived from one or the other, or both, of these neural elements. The Schwann cell proved to have highly distinctive morphological characteristics *in vitro* (illustrated in Plate I), as did the tumor cells; and it was concluded that the tumor is of Schwannian origin (Murray, 1939, 1942; Murray and Stout, 1940, 1942b,c). At the same time, entirely independently, Chlopin (1939, 1940, 1943) was proceeding along essentially similar lines, and arriving at the same conclusion. We have found, in some 15 years experience with cultivating the neurilemoma, that this tumor has such a distinctive aspect *in vitro* that tissue cultures may be used for diagnosis. By this means a small neurilemoma has been found buried in a large thymoma, and the existence of a neurilemoma confirmed in a heretofore unestablished site, the pulmonary parenchyma (Lane, Murray, and Fraser, 1953).

Hemangiopericytoma. The glomus tumor, usually benign and encapsulated, is a complex organoid neoplasm that reproduces in caricature the neuro-myo-arterial glomus, which serves as an arterio-venous shunt mechanism. A conspicuous feature of both the normal and the neoplastic glomus is the sheath of "epithelioid" cells surrounding its endothelium-lined vascular components. The function of these epithelioid cells has been thought to be contractile, operating so that they are able to obliterate the vascular lumen. By a fortunate circumstance the authors were able to obtain for culture a large, infiltrating tumor of the glomus

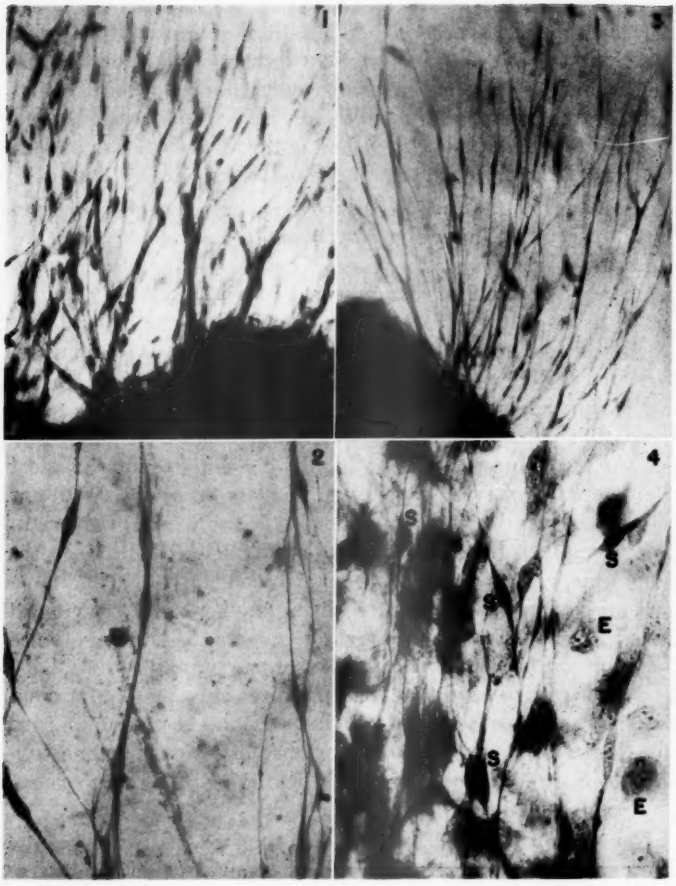


PLATE I. Neurilemoma and normal nerve *in vitro*

FIG. 1. Mediastinal neurilemoma.

FIG. 2. Neurilemoma from popliteal nerve.

FIG. 3. Schwann cells from adult human coeliac nerve.

FIG. 4. Schwann cells (S) and endo-perineural cells (E) from young rat sciatic nerve.

type which had areas in which the epithelioid cells were extraordinarily numerous and close packed. Portions of such areas when explanted dissolved into a reticular outgrowth composed of small spidery, discrete cells with ramose processes, actually very far from "epithelioid" (Murray and Stout, 1942a). When these cells in culture were stained with silver (Bodian method) and were compared with the capillary pericytes (also silver-stained) described by Zimmermann (1923) as occurring in a great variety of anatomical situations in man, there emerged a strong presumption that the two types of cell are identical (Plate II, Figs. 1, 2). The tumor cells often accumulated on capillaries which grew out into the medium (Fig. 3). Zimmermann found a regular transition from the smooth muscle cell of the arteriole to the ramose pericyte which grasps the capillary, and which he believed performs the function of regulating its lumen (a function ascribed similarly to the Rouget cell of the amphibia). In our subsequent study of tumors of this type, we have been impressed by the variety of branching form and the degree to which emphasis may be shifted from the simple bi-polar smooth-muscle type of cell to the extremely ramose pericyte. In the benign, circumscribed glomus tumor, the form adopted by the epithelioid cell *in vitro* is generally closer to that of smooth muscle.

As the result of these tissue culture observations it has been possible to establish a tumor category, designated *hemangiopericytoma,* in which the epithelioid cell or pericyte is the neoplastic element; and to place in it a considerable number of hitherto undiagnosed vascular neoplasms (Stout, 1949).

Similarly, the fortuitous cultivation of a solitary fibrous tumor of the pleura (Stout and Murray, 1942) led to its recognition as a mesothelioma and rescued from the limbo of the misunderstood a whole series of solitary and diffuse tumors of the serous membranes which formerly had masqueraded under some fourteen different and erroneous labels (Stout and Himadi 1951).

Diagnosis

Tumors of nervous derivation. Sympathicoblastoma. As R. G. Harrison (1907) and subsequent workers have observed, the nerve cell when explanted speedily asserts its identity by putting forth neurites; if it is a mono- or bipolar neuron, the slender,

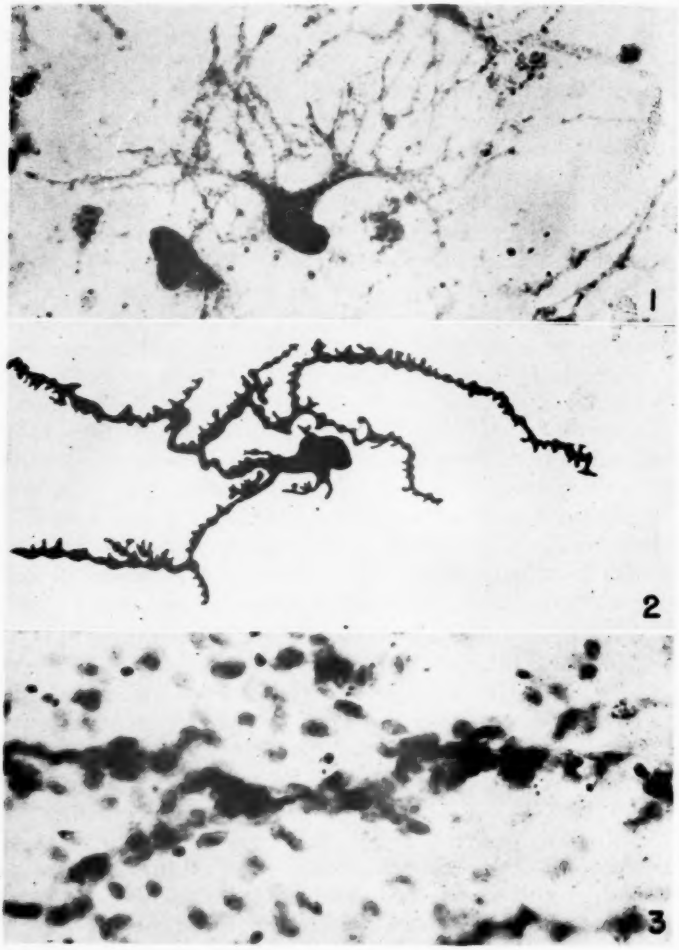


PLATE II. Hemangiopericytoma

FIG. 1. "Epithelioid" cell, or pericyte, *in vitro*.

FIG. 2. Zimmermann's (1923) capillary pericyte. Redrawn.

FIG. 3. Pericytes clustered on capillaries in a culture of hemangiopericytoma.

uniform axon with its pseudopodial tip may reach a considerable length in a few days. The neoplastic neuroblast is no exception to this rule; and since a tear-shaped cell with one or two filamentous processes of 5–10 times its own length presents a striking and distinctive picture, it often happens that the somatic neuroblastoma (or sympathicoblastoma) can be diagnosed within 24 hours in tissue culture, more quickly as well as more surely than by the regular histological methods (Murray and Stout, 1947). The neurites continue to grow and become more numerous (Plate III, Fig. 2), and frequently there develops a tongue- or sheet-like outgrowth of neuroepithelium (Fig. 1), also bearing neurites,—all these characteristics together providing a seemingly infallible test for this tumor derived from embryonal sympathetic neuroblasts. The highly distinctive growth habit of the nerve cells at this stage of development can be used profitably in making a differential diagnosis between the sympathicoblastoma and the Ewing-tumor, the lymphoblastoma and other growths with which it can be confused on clinical or histological grounds. It has also enabled us to pick up sympathicoblastomas in adults, where this tumor is not nearly so malignant as it is in children.

The ganglioneuroma, usually benign, offers little diagnostic difficulty by routine pathological methods. However, it also presents a distinctive picture *in vitro*, producing numerous Schwannian outgrowths as well as some mature, large, multipolar ganglion cells (Murray and Stout, 1948).

An ependymoma is readily recognized in tissue culture, especially if it contains ciliated cells, whose action catches the eye. In any case one may find forms like the lining-cells of the chorioid plexus (Cameron, 1953) as well as ependymal types which retain their long basement processes. *In vitro* observations of this sort have enabled us to confirm a suspected metastasis to the groin of a six year old child, from a dorsally projecting spinal ependymoma which had been excised two years earlier.

Like the Schwann cell, the central neuroglia preserves and intensifies its cellular individuality *in vitro*, as Pomerat (1952) has recently redemonstrated brilliantly in time-lapse moving pictures with phase contrast microscopy. Microglia, astroglia and oligodendroglia come to life vividly and go their appointed rounds. Even in still photographs, the astroblasts of a glioblas-

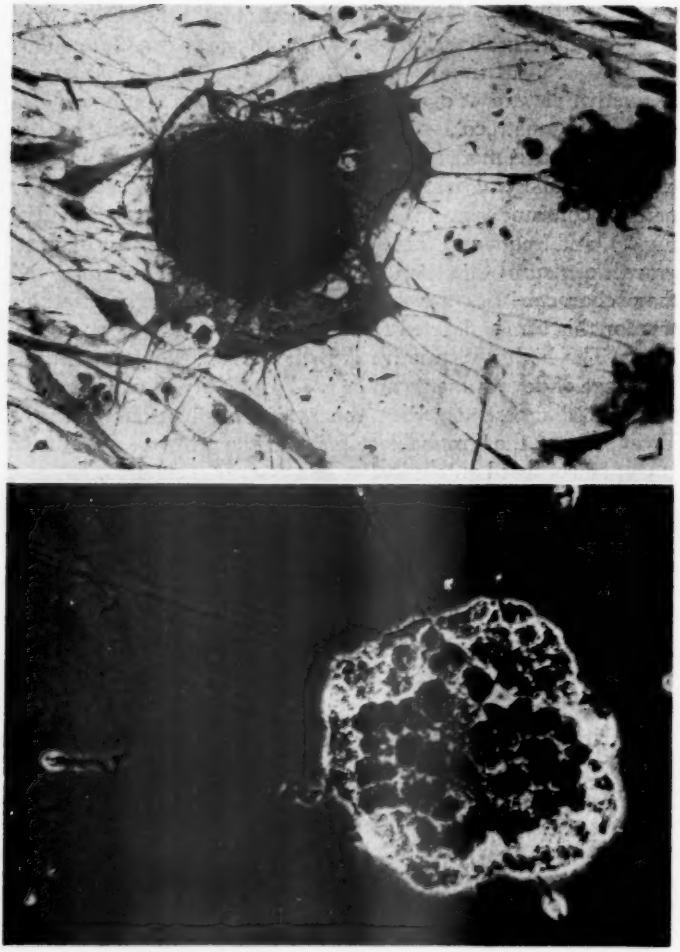


PLATE III. Sympathicoblastoma

FIG. 1. Neurites and neuro-epithelium in culture from a metastatic nodule in a one-year-old child. Bodian's protargol method.

FIG. 2. Living culture (by phase contrast illumination) from a metastasis in a three-year-old child.

toma multiforme may demonstrate their rayed constitution strikingly (Plate IV). We can confirm the early observations of Russell and Bland (1933) on the individuality of form and behavior among intracranial tumor types *in vitro*, and add the suggestion that the tissue culture of these neoplasms is able to provide the most delicate diagnostic criteria.

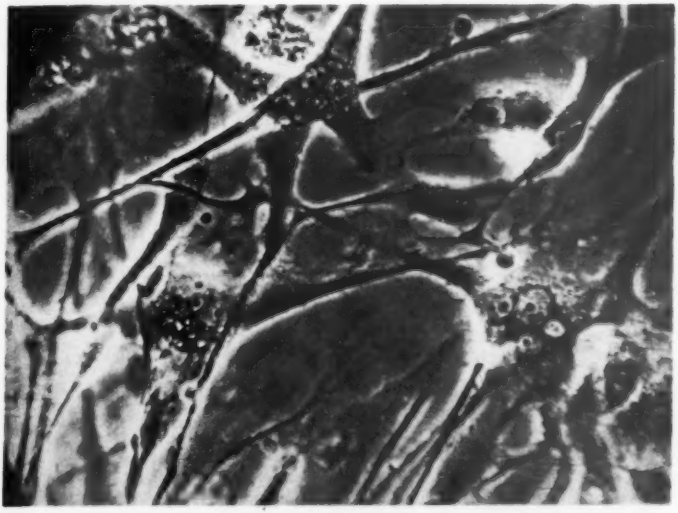


PLATE IV. Rayed astroblasts (multinucleate) in living culture of a glioblastoma multiforme (by phase contrast illumination)

Lymphomas. Into this confused and disputed field the method of tissue culture may shed a little light. It is generally possible by this means to intensify the distinctions between lymphomas of inflammatory origin and the solitary or systemic neoplasms such as lymphocytic lymphosarcoma and reticulum-cell sarcoma. This is not of itself remarkable, but it is of interest that Hodgkin's lymphnodes cultivated *in vitro* invariably align themselves with the inflammatory lymphomas. The lymphocytes of a lymphosarcoma *in vitro* are difficult to distinguish morphologically from those of a normal lymph node (Plate V); normal and neoplastic reticulum cells also are essentially similar in appearance and behavior (Plate VI, Fig. 2). The cells of a Hodgkin's outgrowth

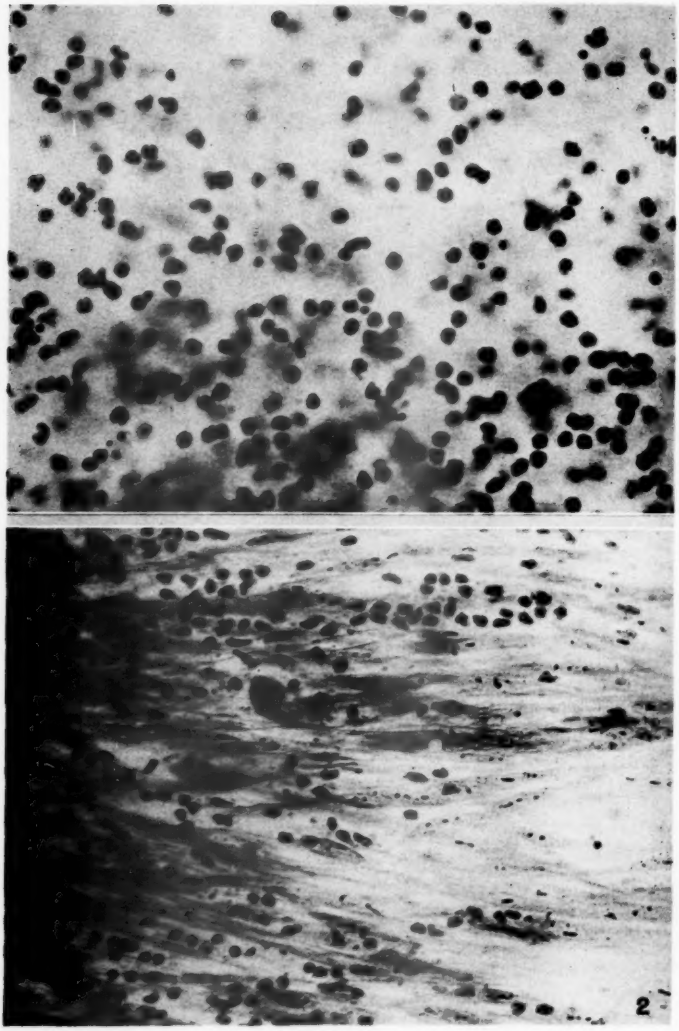


PLATE V. Lymphosarcoma (lymphoblastic type)

FIG. 1. 2-day culture of inguinal node from 11-year-old child.
FIG. 2. 8-day culture from stomach of man 52. Lymphocytes and fibroblasts.

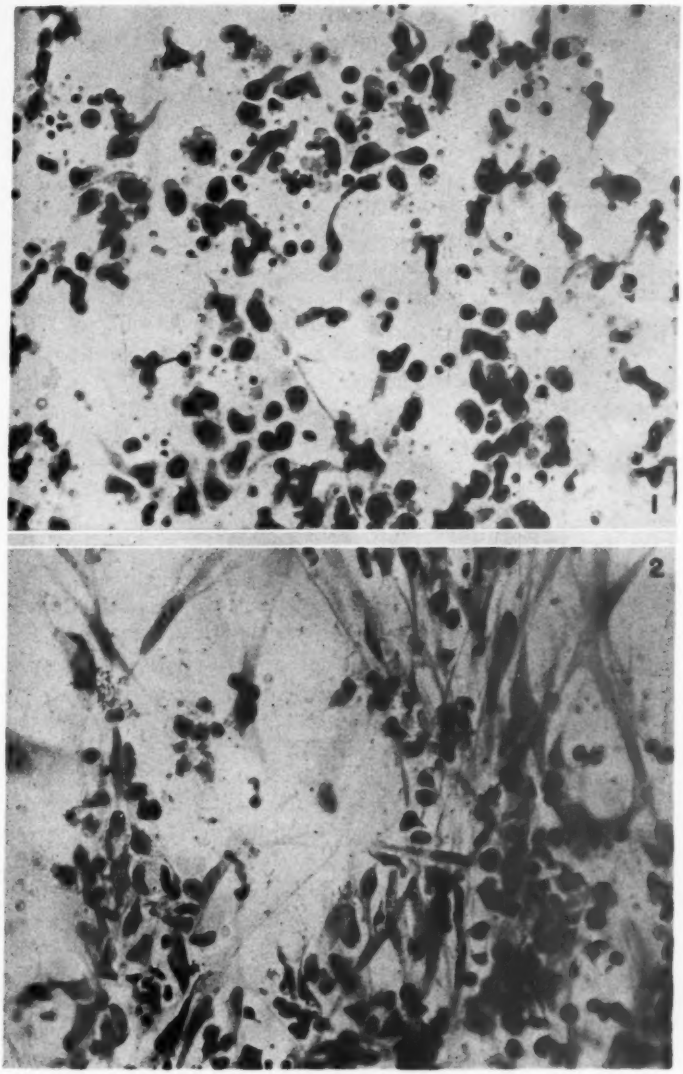


PLATE VI

FIG. 1. 4-day culture from cervical node, Hodgkin's disease.
FIG. 2. 4-day culture from inguinal node, reticulum-cell sarcoma.

however are active, sticky, proteolytic and pleomorphic. A series of forms which appear to be transitional between lymphocyte, reticulum cell, small Dorothy Reed cell, and huge multinucleate cell are everywhere in evidence. An abundance of large, granular, vacuolated cells evolve continuously in these cultures, developing as many as 50 nuclei before becoming pyknotic and disintegrating (Pates VII and VIII).

In cultures from a case of Boeck's sarcoid we have seen certain similarities to the Hodgkin's picture. The indications of inflammation such as cellular activity, stickiness and proteolysis are present, also the metachromatic staining with intravital brilliant cresyl blue, which characterizes Hodgkin's nodes but not lymphosarcoma. But in this situation we find only binucleate cells, relatively small. In fibrosed tubercular nodes as well as in miliary tuberculosis, the inflammatory picture is also present, and in the latter case the fibroblasts are hypertrophic and hyperchromatic: this does not commonly occur in fibroblasts from Hodgkin's nodes. A feature of fibrosed tubercular node is the frequent outgrowth of capillary tubes. In tubercular nodes the giant cells tend to be mononuclear; when multinucleate they rarely are granular and vacuolated as is the rule in Hodgkin's cultures. However, in miliary tuberculosis this is sometimes seen (Plate IX). The disease organism can be demonstrated in the t.b. cultures with Ziehl-Neelsen staining. Nodes with early carcinomatous metastases often appear inflammatory *in vitro*.

The sharp dichotomy between the lymphosarcomas and the inflammatory lymphomas in culture can often be of diagnostic assistance in conjunction with clinical and routine pathological findings. Practically, it has frequently been of value to us in detecting Hodgkin's disease. Of considerable interest also are the theoretical implications of these observations on the inflammatory nature of Hodgkin's disease, which are consistent with those of Bostick, 1952; Hoster *et al.*, 1947 and others. Our observations indicate that differences among outgrowths from "types" (Hoster and Reiman, 1950) of Hodgkin's disease are quantitative rather than qualitative.

[The foregoing has been offered only as an *illustrative* account of the uses of tissue culture in the classification and diagnosis of tumors. For a fuller discussion and review, the reader is referred to a forthcoming chapter by Murray and Stout (Pack and Ariel, in press).]

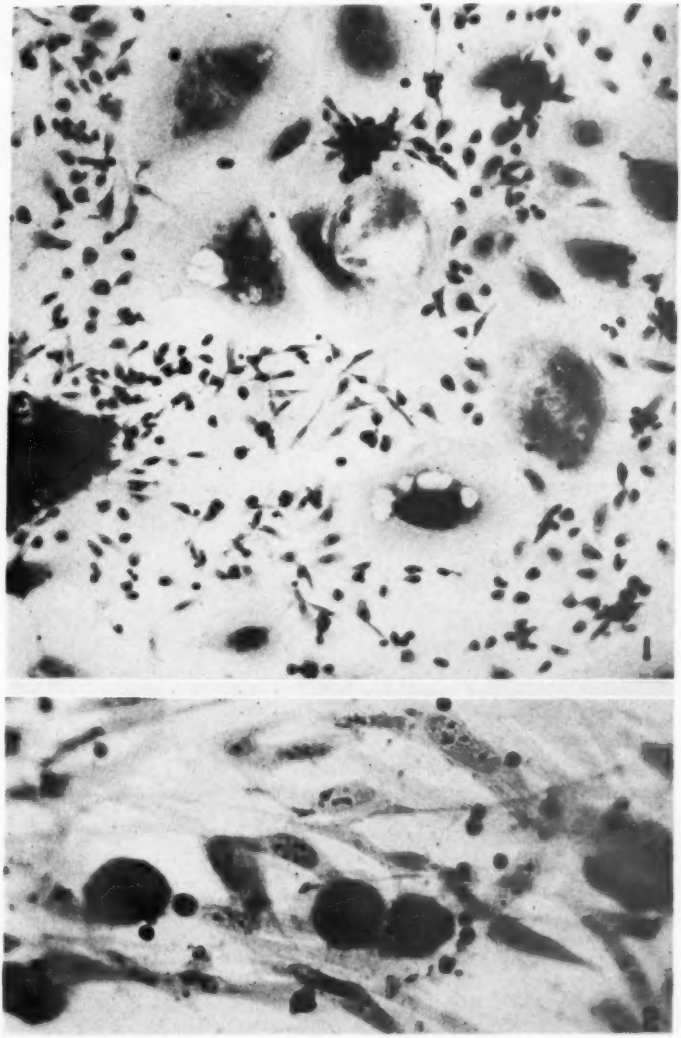


PLATE VII. Hodgkin's disease

FIG. 1. 8-day culture from cervical node. Note variety of cell size, number of nuclei, pyknosis and vacuolation.

FIG. 2. 9-day culture from mediastinal mass. Binucleate cells resembling Dorothy Reed cells as these appear in sections.

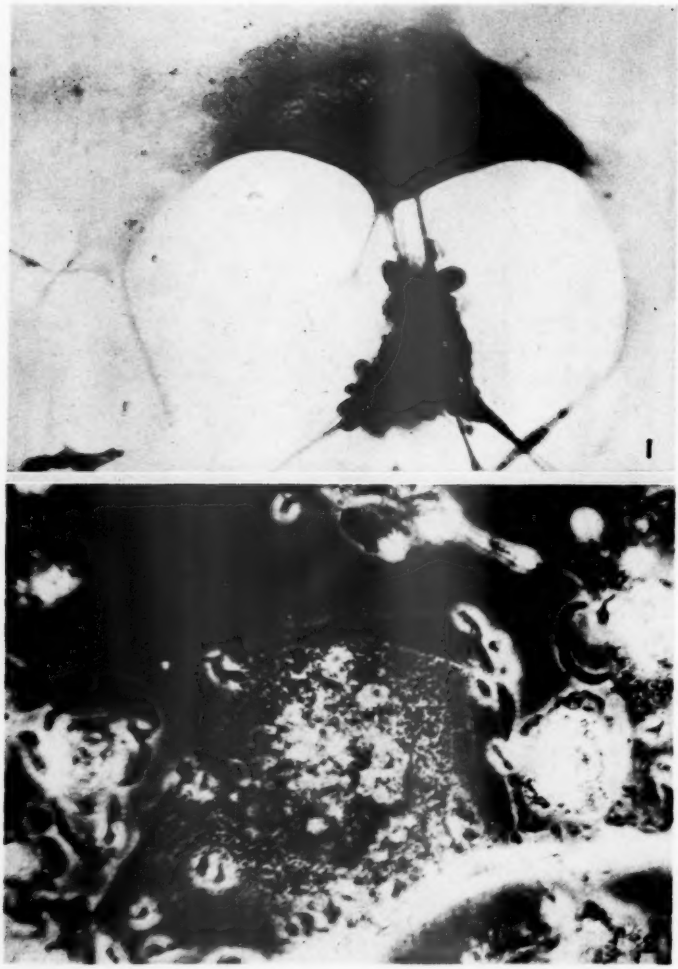


PLATE VIII. Hodgkin's Disease

FIG. 1. Characteristic large multinucleate cell (50 + nuclei) showing granular, vacuolated, intra-cellular mass adjoining liquefied plasma area. Foamy cytoplasm extends to right and left. A mass of agglutinated cells is below.

FIG. 2. Large living cell showing granules and needle-shaped pseudopodia. Smaller cells of the same type are in the field. Phase contrast illumination.

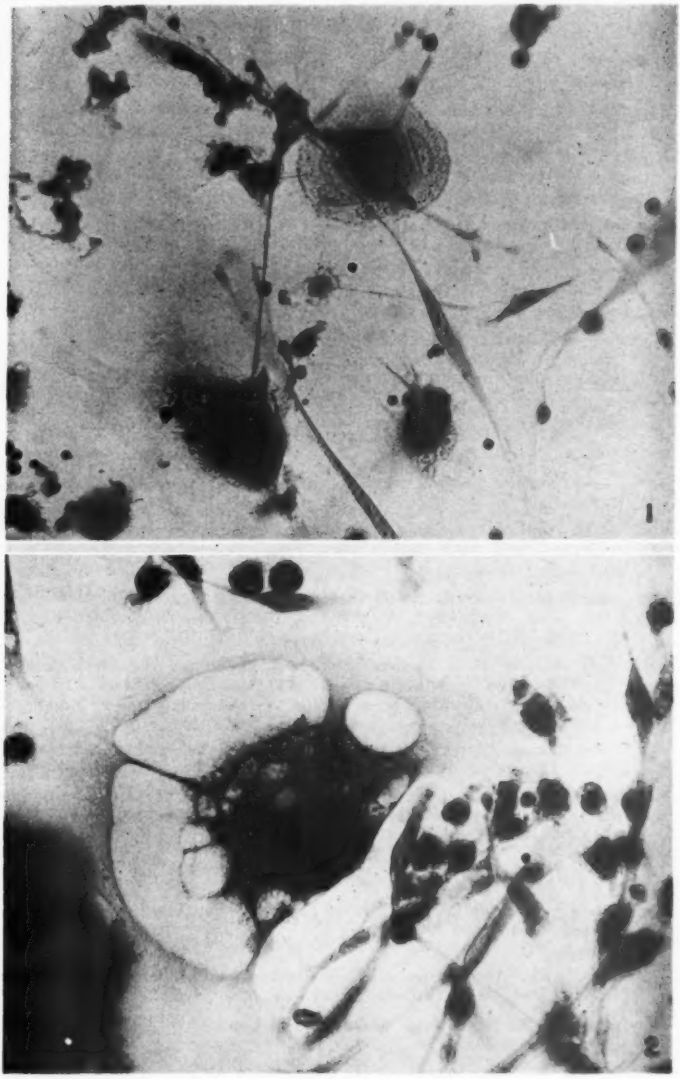


PLATE IX. Tuberculosis

FIG. 1. Fibrosed node from man 29.
FIG. 2. Miliary tuberculosis, girl 12. Large, granular, multinucleate cell like those developing in cultures of Hodgkin's nodes.

REFERENCES

Bloom, W.: Cellular Differentiation and Tissue Culture. *Physiol. Rev.*, 17: 589–617, 1937.

Bostick, W. L.: The Status of the Search for a Virus in Hodgkin's Disease. In: Viruses as Causative Agents in Cancer. *N. Y. Acad. Sci. Annals*, 54: 1162–1176, 1952.

Cameron, G.: Secretory Activity of the Chorioid Plexus in Tissue Culture. *Anat. Rec.*, 117: 115–125, 1953.

Chlopin, N. G.: Über das Wachstum und die Organisationsfähigkeit einiger Epithelgewebe ausserhalb des Organismus. *Zeitschr. Krebsforsch.*, 37: 256–276, 1932.

————: Über einige Probleme der modernen Histologie. *Zeitschr. mikr. anat. Forsch.*, 34: 23–42, 1933.

————: Experimentell-histologische Untersuchungen über das Colömepithel. *Acta zool., Stockholm*, 18: 459–499, 1937.

————: On the *In Vitro Cultivation of Peripheral Nerve Fragments. Acad. Sci. URSS Compt. Rend.*, 23: 175–177, 1939.

————: Ueber das Verhalten und des Wachstum peripherer Nerven in Explantat. *Arch. Russes Anat.*, 23: 171–184; 223–226 Summary, 1940.

————: On the Cultivation of Neurogenic Tumors of Man Outside the Organism. *Acad. Sci. URSS Compt. Rend.*, 41: 132–134, 1943.

Fell, H. B.: Methods for Study of Organized Growth *In Vitro*. Introduction. In: VISSCHER, M. B., ed., *Methods in Medical Research*. Chicago, The Year Book Publishers, Inc. [1948–1951]. [4] v.; 4: 233–237, 1951.

Harrison, R. G.: Observations on the Living Developing Nerve Fiber. *Soc. Exper. Biol. Med. Proc.*, 4: 140–143, 1906–1907; *Anat. Rec.*, 1: 116–118, 1906–1908.

Hoster, H. A., and M. S. Reiman: Studies in Hodgkin's Syndrome. X. The Morphology and Growth Patterns of Explanted Cells Cultivated *In Vitro*. *Cancer Research*, 10: 423–430, 1950.

Hoster, H. A., J. W. Riddle, M. D. Heise, M. S. Flower, M. E. Shanley, B. J. Welshimer, and C. A. Doan: Studies in Hodgkin's Syndrome. VI. Clinical and Etiological Studies. *Ohio State Med. J.*, 43: 721–724, 1947.

Kredel, F. E.: Tissue Culture of Intracranial Tumors. With a Note on the Meningiomas. *Am. J. Path.*, 4: 337–340, 1928.

Lane, N., M. R. Murray, and G. C. Fraser: Neurilemoma of the Lung Confirmed by Tissue Culture. Report of a Case. *Cancer, New York*, 6: 780–785, 1953.

Leighton, J.: A Sponge Matrix Method for Tissue Culture. Formation of Organized Aggregates of Cells *In Vitro*. *Nat. Cancer Inst. J.*, 12: 545–561, 1951.

Marcuse, P. M.: Application of Neotetrazolium to Outgrowths of Human Neoplasms in Tissue Culture. *Texas Rep. Biol. Med.*, 12: 126–133, 1954.

Maximow, A. A.: Relation of Blood Cells to Connective Tissues and Endothelium. *Physiol. Rev.*, 4: 533–563, 1924.

Murray, M. R.: Demonstration of Schwannian Origin of Tumors of the Nerve Sheaths. *Arch. Neurol. Psychiat., Chicago*, 42: 1175, 1939.

————: Comparative Data on Tissue Culture of Acoustic Neurilemomas and Meningiomas. *J. Neuropath. Exper. Neurol.*, 1: 123–124, 1942.

————: Tissue Culture Procedures in Medical Installations: In: VISSCHER, M. B., ed., *Methods in Medical Research.* Chicago, The Year Book Publishers, Inc. [1948–1951]. [4] v.; 4: 211–216, 1951.

————, and A. P. Stout: Schwann Cell Versus Fibroblast as the Origin of the Specific Nerve Sheath Tumor. Observations Upon Normal Nerve Sheaths and Neurilemomas *In Vitro. Am. J. Path.*, 16: 41–60, 1940.

————, and ————: The Glomus Tumor. Investigation of Its Distribution and Behavior, and the Identity of Its "Epithelioid" Cell. *Am. J. Path.*, 18: 183–203, 1942a.

————, and ————: Demonstration of the Formation of Reticulin by Schwannian Tumor Cells *In Vitro. Am. J. Path.*, 18: 585–593, 1942b.

————, and ————: Characteristics of Human Schwann Cells *In Vitro. Anat. Rec.*, 84: 275–293, 1942c.

————, and ————: Distinctive Characteristics of the Sympathicoblastoma Cultivated *In Vitro:* A Method for Prompt Diagnosis. *Am. J. Path.*, 23: 429–442, 1947.

————, and ————: A Sympathetic Ganglioneuroma Cultivated *In Vitro. Cancer*, 1: 242–247, 1948.

Pack, G. T., and I. M. Ariel: *The Treatment of Cancer and Allied Diseases.* Ed. 2, Paul B. Hoeber, Inc., New York (in press).

Pomerat, C. M.: Dynamic Neurogliology. *Texas Rep. Biol. and Med.*, 10: 885–913, 1952.

Russell, D. S., and J. O. W. Bland: A Study of Gliomas by the Method of Tissue Culture. *J. Path. Bact.*, 36: 273–283, 1933.

Stout, A. P.: Hemangiopericytoma. *Cancer*, 2: 1027–1054, 1949.

————: Tumors of the Soft Tissues. In: Atlas of Tumor Pathology. Washington, D. C. Armed Forces Institute of Pathology, Section II, Fascicle 5, 138 pp., 1953.

————, and G. M. Himadi: Solitary (localized) Mesothelioma of the Pleura. *Ann. Surg.*, 133: 50–64, 1951.

————, and M. R. Murray: Localized Pleural Mesothelioma; Investigation of Its Characteristics and Histogenesis by Method of Tissue Culture. *Arch. Path.*, 34: 951–964, 1942.

Timofeevskii, A. D.: Tumor Origin as Determined by Tissue Culture. *Am. Rev. Soviet Med.*, 4: 106–110, 1946–1947.

Verocay, J.: Multiple Geschwülste als Systemerkrankung am nervösen Apparate. Festschrift Hans Chiari aus Anlasz seines 25 Jährigen Professoren-Jubiläums gewidmet. W. Braumüller, Wien und Leipzig, 378–415, 1908.

Weiss, P.: Principles of Development. Henry Holt & Company, New York. 1939.

Zimmermann, K. W.: Der feinere Bau der Blutcapillaren. *Ztschr. f. Anat. u. Entwicklungsgesch.*, 68: 29–109, 1923.

TISSUE CULTURE OF HUMAN BREAST CARCINOMA AND NORMAL BREAST EPITHELIUM*

MARY FAITH ORR AND BARTON MCSWAIN†

This is a report on the results of tissue cultures of an unselected series of primary carcinomas of the human female breast, with comments on the cultural procedures and certain characteristics of malignant epithelium as compared to normal breast epithelium.

Some information has been obtained on the cultivation of carcinomas of the human breast (Cameron and Chambers, 1937; Coman, 1942; Royle, 1946), but extensive studies are lacking.

Early results in this laboratory made the pursuit of such an investigation seem worthwhile. The cultivation of normal breast tissue has also shown promising results.

Materials and Methods. Media and manipulation of cultures were kept as constant as possible.

Specimens of primary carcinomas of the breast were obtained for tissue culture. The tissue was immediately cut into fragments about 1 to 2 mm. square, being careful to dissect away fat and large pieces of connective tissue. The fragments were washed in rapid changes of balanced salt solution until the solution was clear. Normal breast tissue was treated in the same manner.

Fresh heparinized chicken plasma and ten-day old embryonic extract were used for the clot. A nutrient solution composed of 50 per cent Hanks' salt solution, 47 per cent pleural or ascitic fluid, 3 per cent embryo extract, and 500 units penicillin G per cc. was used. The pH of all media was 7.4 to 7.6.

The Carrel flask (D 3.5) and roller tube methods were chosen for this study. Preparation of the Carrel flask consisted of planting six fragments of tissue in a clot composed of equal parts of chicken plasma and embryo extract. After allowing the clot to become firm, 1 ml. of nutrient fluid was introduced and the flask closed with a rubber stopper. Roller tubes were made by clotting three

* From the Department of Surgery, Vanderbilt University School of Medicine, Nashville, Tenn. Presented at the Eighth Annual Cancer Symposium, April, 1954, at the M. D. Anderson Hospital, Houston.

† The authors express their appreciation to Mr. Wendell Gillett for preparation of photomicrographs.

or four fragments on a No. 1, 12 by 50 mm. coverglass. This preparation was placed in a 16 by 150 mm. pyrex test tube. Two mls. of nutrient fluid were added. One half of the tubes, closed with rubber stoppers, were put into a roller drum apparatus rotating ten times an hour. One half of them were slanted in a fixed position. All cultures were incubated at 36° C.

After the first signs of outgrowth were observed, the nutrient fluid was renewed routinely once a week. If metabolic activity of a culture of fibroblasts caused the media to become acid rapidly, the fluid was changed more often.

Where liquefaction of the clot occurred, a patch was made by removing the fluid and applying new plasma and embryo extract.

Subculturing depended upon the type of outgrowth. If most of the cells were fibroblasts, subculturing was done monthly. Epithelial outgrowth was very seldom disturbed.

Carrel flasks were used for maintaining cultures for long periods, while roller tube cultures were primarily used for staining at various intervals.

A staining method devised by Jacobson and Webb (1952) employing Giemsa and May-Greenwald stains was used.

Results and Comments

The following table gives a summary of the tissue culture results:

TABLE 1

Tissue Culture Results

Tissue	Epithelium and Fibroblasts	Fibroblasts Only	Poor Outgrowth or No Outgrowth	Total
Adenocarcinoma	15	6	10	31
Duct cell Carcinoma	9	3	0	12
Carcinoma, unclassified	5	1	5	11
Normal breast tissue	2	1	0	3

In a majority of the cases the results obtained were quite satisfactory; in others, they were inadequate. The common causes for poor results were contamination, trauma, and removal of an unsatisfactory portion of the tumor. In some instances the explants

caused rapid liquefaction of the clot and were lost. However, when sterile specimens of cellular, non-necrotic portions of tumors were available, good results were obtained with the cultural conditions described. All of the tumors produced the same types of outgrowth.

One hundred explants were cultured from each tumor. About 80 per cent of these showed an outgrowth of cells. Outgrowths were usually evident between the fourth and sixth day of cultivation. Both Carrel flasks and roller tubes produced similar results.

Fibroblastic (Fig. 1) outgrowth presented the typical picture of a connective tissue culture. They were masses of spindle-shaped cells which existed and proliferated more rapidly and much longer than the epithelial cells. Their presence or absence did not affect the epithelial formations in initial cultures.

Normal breast tissue produced epithelial sheets (Fig. 2) made up of cells with lightly stained, inconspicuous cytoplasm. The nuclei varied somewhat in size but had smooth regular membranes, and each contained one or more small nucleoli. Marked liquefaction of the clot was not observed.

While epithelial formations such as cords, tongues (Fig. 3), broken sheets, and isolated groups of cells occurred in outgrowths from tumor tissue, epithelium appeared frequently as sheets of cells (Fig. 4). These sheets were often relatively free from fibroblasts, tended to liquefy the clot and adhere to the glass. They could be maintained in good condition for one to four months by changing the nutrient fluid regularly. Cytologically (Fig. 5) these cells stained much more densely than those of normal epithelium. They varied in size, assumed various shapes, and tended to separate. The nuclei were of different sizes and frequently had irregular membranes. There was an increase in nucleolar material which was variable in amount and shape. Only a few of the outgrowths of epithelium showed any frequency of mitosis.

Summary. The method of successfully culturing carcinomas of the human female breast has been described.

Normal breast tissue has been successfully cultured under the same conditions.

The proliferation of both epithelial cells and fibroblasts has been described.

Cytologic differences between epithelium of normal breast and tumor tissue have been described.

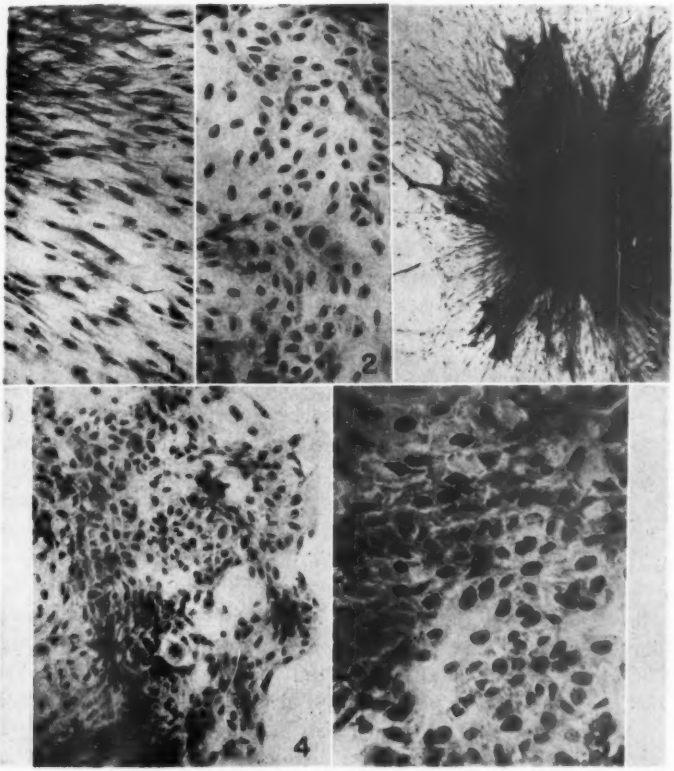


FIG. 1. An outgrowth of fibroblasts from a tissue culture of a specimen of duct-cell carcinoma of the breast (34009). X 150.

FIG. 2. Epithelial outgrowth from a tissue culture of normal breast tissue (33441). X 150.

FIG. 3. A mixed outgrowth of tongues of epithelium and fibroblasts from a tissue culture of an adenocarcinoma of the breast (53–215). X 8.

FIG. 4. An outgrowth of a sheet of epithelial cells from a tissue culture of an adenocarcinoma (33590). X 75.

FIG. 5. An area of the sheet of epithelial cells in figure 4 photographed at higher magnification. X 300.

REFERENCES

Cameron, Gladys, and R. Chambers: Neoplasm Studies III. Organization of Cells of Human Tissues in Tissue Culture. ***The Am. Jour. of Cancer***, 30: 115–129, 1937.

Coman, D. R.: Human Neoplasms in Tissue Culture. ***Cancer Research***, 2: 618–625, 1942.

Jacobson, W., and M. Webb: The Two Types of Nucleoproteins During Mitoses. ***Expt. Cell Research***, 3: 363–383, 1952.

Royle, J. G.: Some Cultural and Cytological Characteristics of Human Tumors *In Vitro*. ***Cancer Research***, 6: 225–229, 1946.

THE MACROMOLECULAR NUCLEOPROTEIN PARTICLES OF NORMAL RAT LIVER AND AZO-DYE INDUCED LIVER TUMORS

MARY L. PETERMANN*

The presence in normal cytoplasm of nucleoprotein particles in the size range of macromolecules (about 20 mμ) has long been recognized by virologists. Ultracentrifugal analysis has shown the sedimentation rates of these substances to be about 70S (Taylor, Sharp, and Woodhall, 1943). Kahler and Bryan (1943) studied aqueous extracts of mouse mammary tumors and Jensen rat sarcoma. They found a principal boundary with a sedimentation rate of 60S in the mammary tumors, while the preparation from Jensen rat sarcoma had a sedimentation rate of only 43S.

Our first studies were carried out on mouse spleen (Petermann, Mizen, and Hamilton, 1952). When cytoplasmic extracts made in 0.88 M sucrose were centrifuged at 152,000 x g. long enough to sediment 90 per cent of the pentose nucleic acid (PNA), and the resuspended sediment was examined in the analytical ultracentrifuge, a number of well-defined boundaries were observed, with sedimentation rates of about 70, 50, 40, 34, and 27S. These were called, for convenience, A, B, C, D and E. Components B, C and E were present in much higher concentrations in both spontaneous and transplanted leukemic spleen. Similar substances have since been noted on ultracentrifugal analysis of liver, pancreas, and several tumors (Petermann, Mizen, and Hamilton, 1953, 1954), and observed by electron micrography of thin sections of many organs (Palade, 1953).

The ultracentrifuge pattern of the macromolecules of liver differs somewhat from that found for spleen. The B, C and E boundaries are similar but no D boundary is seen. The A boundary is much larger, and small amounts of faster-sedimenting components, A″ and A′, with sedimentation rates of about 107 and 90S, are present.

---

* From the Laboratories of the Sloan-Kettering Institute and the Sloan-Kettering Division of Cornell University Medical College, New York. This work was supported by grants from the Atomic Energy Commission (Contract AT(30–1)–910) and the National Cancer Institute of the United States Public Health Service (Grant C–471). Presented at the Eighth Annual Cancer Symposium, April, 1954, at the M. D. Anderson Hospital, Houston.

Electrophoretic analysis of either spleen or liver preparations shows four components of high mobility, which have been tentatively identified with the components observed in the ultracentrifuge (Petermann, Hamilton, and Mizen, 1954). In veronal-chloride buffer at pH 8.6 and ionic strength 0.10 component B appears to have a mobility of —8.0, C of —6.9, and E of —9 or 10 cm.^{2}/sec./volt x 10^{-5}. No free PNA (mobility —13) is seen. Similar patterns are obtained in borate buffer.

The study of these macromolecular nucleoproteins has been technically difficult, because of their lack of stability. When preparations are made in buffered saline the ultracentrifuge boundaries are much smaller than those obtained in sucrose. When the particles are sedimented in sucrose, then resuspended in buffer and allowed to stand overnight in the cold, the ultracentrifuge pattern shows a decrease of about 50 per cent in the concentrations of C and E (Petermann, Mizen, and Hamilton, 1952). All the components are very unstable on dialysis; the ultracentrifuge boundaries are 25 per cent smaller after only three hours of dialysis at pH 8.6, and completely gone after 16 hours. The electrophoretic analyses have to be made on undialyzed preparations (Petermann, Hamilton, and Mizen, 1954). Particles resuspended in 0.88 M sucrose are moderately stable in the cold, lasting about five days, and appear to be stable for months in the deep-freeze cabinet.

These particles are very rich in PNA. None of the fractions obtained so far have been sufficiently pure for precise chemical analysis; but in one electrophoresis experiment, made in borate buffer, two samples of the faster-moving components, free of microsomes and hemoglobin, were withdrawn from the ascending boundary. One sample, a mixture of B and E, was 50 per cent PNA by chemical analysis. The other, which contained only E, was too dilute for chemical analysis, but its ultraviolet absorption curve showed it to be even richer in PNA. A second approach has been to estimate the per cent of the total protein and PNA in a preparation accounted for by the macromolecules, and to plot this against the PNA content of the preparation. When such a plot is extrapolated to 100 per cent macromolecules it intersects the ordinate at 47 per cent PNA (Petermann, Hamilton, and Mizen, 1954).

The average rat liver contains about 18 mg. of microsomes and 3.6 mg. of macromolecules per gram (Petermann, Mizen, and Hamilton, 1953). If the microsomes are ten per cent PNA, their total PNA content would be 1.8 mg. If the macromolecules are 47 per cent PNA, their PNA content would be 1.7 mg. The macromolecules would, therefore, account for 49 per cent of the submicroscopic PNA of the liver cytoplasm.

In attempts to purify the macromolecules the best guide has been this comparison of total macromolecules, measured in the ultracentrifuge, with total protein + PNA. A number of fractionation procedures have been employed. The particles are precipitated by protamine (Warren, Weil, Russ, and Jeffries, 1949), but have not been recovered from the precipitate. They can be precipitated by acetic acid at pH 5.6 and redissolved at pH 8.6, but this does not remove the microsomes. Precipitation at pH 6.0 with 17 per cent ethanol or at pH 6.3 with 30 per cent ethanol is also ineffective. Precipitation with calcium is much more effective, since a large proportion of the precipitated microsomes fail to redissolve; and if the precipitation is carried out at pH 8.0 complex formation between the particles and hemoglobin can be avoided.

Removal of the microsomes by differential centrifugation in 0.88 M sucrose is inefficient. Although the microsomes are much larger than the macromolecules they do not sediment much faster. This can be ascribed to their lower density, the result of their high lipid content (Barnum and Huseby, 1948). A centrifugal force sufficient to remove most of the microsomes results in a 50 per cent loss in component B. Attempts have therefore been made to utilize these differences in density. The macromolecules are so dense they will still sediment, although very slowly, in 2.05 M (70 per cent) sucrose. When an 0.88 M sucrose preparation is layered over 2.05 M sucrose, and centrifuged for 20 hours at 142,000 x gravity, the macromolecules collect in the 2.05 M sucrose, while most of the microsomes remain in the 0.88 M sucrose. Again, here, if the pH has been adjusted to 8.0 the hemoglobin remains in the upper layers.

In our studies of the macromolecular nucleoproteins in azo-dye induced liver tumors of the rat, four groups of Sherman rats have been used. The first group, which received no dye, included rats

on a stock diet of Purina chow pellets; rats on a rice and carrot diet (Sugiura and Rhoads, 1942) for four months, followed by a stock diet for three months; and rats on the rice and carrot diet for 9 months. Group 2 was made up of animals which were on the rice and carrot diet + 0.06 per cent of p-dimethylaminoazobenzene (Sugiura and Rhoads, 1942) for eleven months, but which failed to develop tumors. Three analyses of the grossly normal portions of tumorous livers were also included. Group 3 included the rats on the diet + dye regime which developed hepatomas, and group 4 included those which developed cholangiomas.

Methods. The first experiments were carried out by a technique similar to that used for analyses of mouse spleen (Petermann, Mizen, and Hamilton, 1952). The livers were not perfused, and the sedimented particles were resuspended in 2.5 ml. of buffer per gram of liver pulp. The later experiments were carried out by the technique used in the regenerating liver studies. After thorough perfusion with saline, the livers were removed and chilled on ice. The firmest portions of the tumors were dissected out for analysis and samples of all the tumors and the livers from rats which had received dye were fixed in ten per cent formalin, stained with hematoxylin and eosin, and examined by a pathologist. The tissue was forced through a mincer, and the resulting pulp homogenized.

The per cent of cells broken was determined by differential counts on smears of the homogenate.

Since the tumor cells are more resistant than normal liver cells, frequently only about eighty instead of ninety per cent of them were broken. In each experiment, therefore, the analytical results have been corrected for the per cent of cells not broken. Total cell counts were made on the homogenates in a haemocytometer. The preparation and analysis of the particles were the same as for the regenerating liver experiments, except that the mitochondria supernatants were shell-frozen and stored in the deep freeze cabinet for varying periods of time. Just before the preparative ultracentrifugation the material was quickly thawed by rotating the bottle in water at 37° until thawing was almost complete. This procedure is much more convenient than carrying out the whole experiment in one day, and in our experience has caused no changes in the particles.

Nitrogen analyses were made by a semi-micro Kjeldahl procedure, and the results were corrected for the nitrogen content of the veronal buffer used.

Results

Typical ultracentrifuge patterns obtained on normal liver and on liver tumors are shown in figure I. The pictures in the left hand column were taken after about four minutes at 37,020 RPM. In normal liver there is a large, spreading boundary (M), which is accompanied by turbidity, and is caused by the sedi-

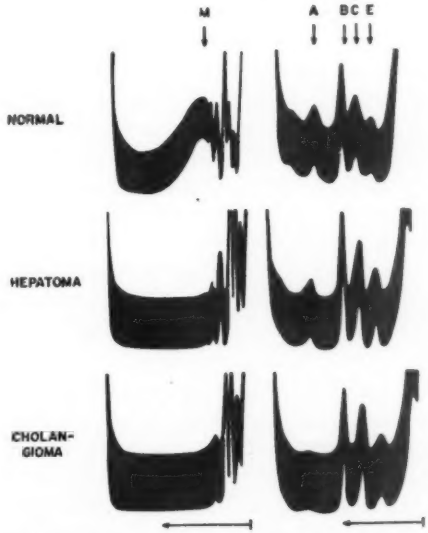


FIG. I. Ultracentrifuge patterns of particles from normal rat liver and liver tumors. Left column, pictures taken after four minutes at 37,020 RPM. M = microsome boundary. Right column, pictures taken after 16 minutes at 37,020 RPM. A, B, C and E are the various macromolecular components.

mentation of the microsomes. In the liver tumors this boundary is much smaller, and the total nitrogen of the preparation is also considerably reduced. The very sharp boundaries in these early pictures represent the various macromolecular components. The pictures in the right hand column were taken after about 16 minutes. The microsome boundary is gone and the macromole-

cule boundaries are separated from one another. In normal liver component B predominates, and the amounts of C and E are relatively small. Both types of tumor show marked elevations in the amounts of C and E.

The amounts of B, C, and E in the various types of livers studied are shown in figure II. Here the ordinates represent the amounts of B, C, and E per gram of liver pulp, and the abscissa is the average cell weight (the reciprocal of the number of cells per gram). For the normal livers, the amount of B per gram is directly proportional to cell size. The same relationship holds for the values found for the non-tumorous livers (or parts of livers) of the animals that received the rice-carrot diet plus the dye. The pathological diagnoses on these livers included, in order of decreasing cell size, one normal, one "essentially normal, but with hyperchromatic nuclei," two cirrhosis, one benign hepatoma, and one "cirrhosis which foci of bile duct proliferation."

In both types of tumors the cells were very small, and the relationship between the amount of B and cell size no longer seemed to hold; the concentration of B was about twice what would be expected for such small cells. Two observations made on regenerating liver, at a time just preceding the most rapid cell division, have also been included. Although the cells are large, the concentration of B is about equal to that expected in cells half their size.

When component C is plotted against cell size the concentrations in the normal livers appear to be independent of cell size, and the non-malignant azo-dye values are quite similar. The values for the tumors, on the other hand, are about fifty per cent higher, and values for regenerating liver again resemble those found in the tumor cells rather than in the non-dividing cells.

The distribution of component E resembles that of C, with a slightly lower concentration in the smaller cells, from both normal and dye-fed animals, and high values in the tumors and regenerating liver.

The amounts of B, C and E per cell are shown in figure III. Here the correlation between the amount of B per cell and cell size is striking, in the normal livers, the intermediate group, and the tumor cells, although the values for tumor cells are about twice what would be expected for normal cells of this size. When

the values for regenerating liver are divided in two they resemble those found for the smallest normal cells. The amount of C per cell is apparently also dependent on cell size, for both the normal livers and the dye-fed group. The values for the tumors are as great as for the normal cells, although the tumor cells are so much

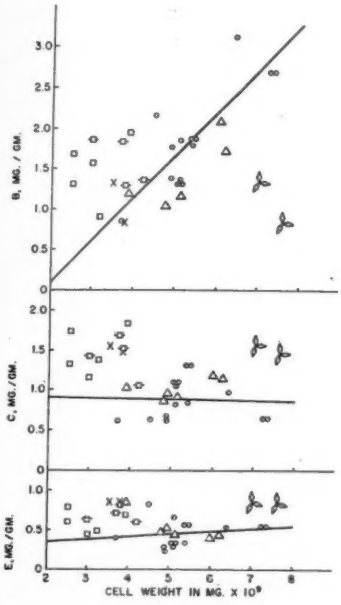


FIG. II. The concentrations of components B, C and E, in mg. per gram of liver pulp, as a function of the average cell weight. O = normal liver; △ = diet + azo-dye, not malignant; -□- = hepatoma; □ = cholangioma; ◇ = regenerating liver, two days after partial hepatectomy; X = same values plotted for cells half the size. The lines were drawn by the method of least squares, including only the normal points.

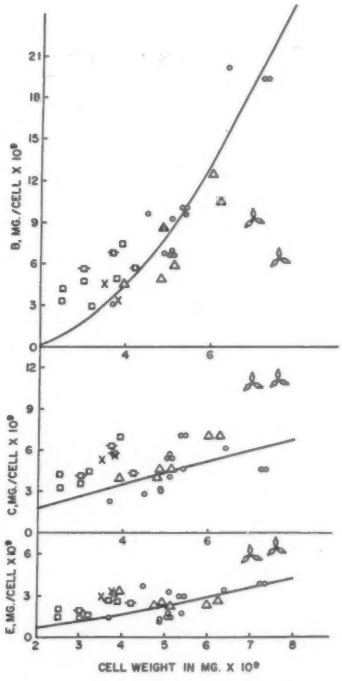


FIG. III. The concentrations of components B, C and E in mg. per cell, as a function of the average cell weight. O = normal liver; △ = diet + azo-dye, not malignant; -□- = hepatoma; □ = cholangioma; ◇ = regenerating liver after two days; X = same experiments, calculated for cells half the size. The lines were calculated from the lines in figure II, and represent only the normal points.

smaller. When the values for regenerating liver are divided by two they again resemble those found for the tumor cells. The amounts of E per cell are generally about half those found for C, and show a similar elevation in the tumor cells and in regenerating liver.

Discussion

When studies on liver tumors were initiated the finding of a decreased concentration of component B was puzzling, since in leukemic spleen B, as well as C and E, was present in greater than normal concentration (Petermann, Mizen, and Hamilton, 1952). This difference may now be ascribed to the effect of cell size; in leukemic spleen the number of cells per gram is reduced to about two-thirds the normal value (Mizen and Petermann, 1952), so the average leukemic cell must be considerably larger than the average normal cell. Since it is also malignant, both factors are additive, and the result is a marked elevation in the concentration of B.

The type of ultracentrifuge pattern obtained in liver tumors, with C present in greater concentration than B, has also been observed in the Jensen sarcoma of the rat and in the Ehrlich ascites tumor of the mouse (Petermann, Mizen, and Hamilton, 1954). In regenerating liver it was seen only during the period of most raid cell division. One is therefore tempted to suggest that C is connected in some way with cell division, while B is involved in cell function.

In an effort to answer some of these questions, a study of rat liver in various physiological conditions is now under way in this laboratory. A summary of the rersults to date is shown in table 1. In each case the values have been compared to those expected for normal liver cells of the same size. From these results one can conclude only that the various macromolecular components observed in the ultracentrifuge represent cell constituents which can vary independently, according to the physiological state of the liver. That similar substances are of widespread occurrence in nature is indicated by their presence in microorganisms (Schachman, Pardee, and Stanier, 1952; Siegel, Singer, and Wildman, 1952) and green leaves (Eggman, Singer, and Wildman, 1953.)

TABLE 1

Changes in the Nucleoprotein Content of Rat Liver from the Normal Adult Values for Cells of the Same Size

Liver	Microsomes	A	B	C	E
Starved 48 hours	0	—	0	—	0
Young,* normal	0	0	0	—	0
Young,* cortisone† ...	†	—	—	‡	0
Regenerating, 2 days..	—	—	—	†	†
Regenerating, 4 days..	—	—	0	0	0
Pregnant, 19 days	†	—	†	0	†
Diet+ azo-dye, non-malignant	0	0	0	0	0
Hepatoma	—	0	†	†	†
Cholangioma	—	0	†	†	†

* 150 gram rats.
† 25 mg. subcu. daily for 5 days.
‡ Increased to normal adult value.

Acknowledgments

The author thanks Dr. K. Sugiura and Dr. C. J. Kensler for the azo-dye fed and diet control rats; Dr. Sophie Spitz for reviewing the histological controls; Dr. Kensler, for many helpful discussions; and Miss Nancy A. Mizen and Mrs. Mary G. Hamilton for invaluable assistance in all phases of this work.

REFERENCES

Barnum, C. P., and R. A. Huseby: Some Quantitative Analyses of the Particulate Fractions of Mouse Liver Cell Cytoplasm. *Arch. Biochem.*, 19: 17–23, 1948.

Eggman, L., S. J. Singer, and S. G. Wildman: The Proteins of Green Leaves. V. A Cytoplasmic Nucleoprotein from Spinach and Tobacco Leaves. *J. Biol. Chem.*, 205: 969–983, 1953.

Kahler, H., and W. R. Bryan: Ultracentrifugal Studies of Some Complexes Obtained from Mouse Milk, Mammary Tumor, and Other Tissues. *J. Nat. Cancer Inst.*, 4: 37–45, 1943–44.

Mizen, N. A., and M. L. Petermann: Nuclei from Normal and Leukemic Mouse Spleen. III. The Desoxypentose Nucleic Acid Content per Nucleus Calculated from Total Cell Counts. *Cancer Res.*, 12: 727–730, 1952.

Palade, G. E.: *A Small Particulate Component of the Cytoplasm.* Electron Microscope Society of America, Pocono Manor, Pa., November, 1953.

Petermann, M. L., N. A. Mizen, and M. G. Hamilton: An Ultracentrifugal Analysis of the Macromolecular Particles of Normal and Leukemic Mouse Spleen. *Cancer Res.*, 12: 373–378, 1952.

Petermann, M. L., M. G. Hamilton, and N. A. Mizen: Electrophoretic Analysis of the Macromolecular Nucleoprotein Particles of Mammalian Cytoplasm. *Cancer Res.*, 14: 360–366, 1954.

Petermann, M. L., N. A. Mizen, and M. G. Hamilton: The Macromolecular Particles of Normal and Regenerating Rat Liver. *Cancer Res.*, 13: 372–375, 1953.

Petermann, M. L., N. A. Mizen, and M. G. Hamilton: The Macromolecular Nuecleoprotein Particles of Normal and Tumor Tissue. *Proc. Amer. Assoc. for Cancer Research,* 1(2): 37, 1954.

Schachman, H. K., A. B. Pardee, and R. Y. Stanier: Studies on the Macromolecular Organization of Microbial Cells. *Arch. Biochem. Biophys.,* 38: 245–260, 1952.

Siegel, A., S. J. Singer, and S. G. Wildman: A Preliminary Study of the High-Molecular-Weight Components of Normal and Virus-Infected *Escherichia Coli. Arch. Biochem. Biophys.,* 41: 278–293, 1952.

Sugiura, K., and C. P. Rhoads: The Effect of Yeast Feeding Upon Experimentally Produced Liver Cancer and Cirrhosis. *Cancer Res.,* 2: 453–459, 1942.

Taylor, A. R., D. G. Sharp, and B. Woodhall: Macromolecular Components of Normal Embryonic and Adult Brain Tissue. *Science,* 97: 226–227, 1943.

Warren, J., M. L. Weil, S. B. Russ, and H. Jeffries: Purification of Certain Viruses by Use of Protamine Sulfate. *Proc. Soc. Exptl. Biol. Med.,* 72: 662–664, 1949.

RADIATION EFFECTS IN TISSUE CULTURE*

AGNES N. STROUD AND AUSTIN M. BRUES

Tissue culture has proven to be a very useful tool in the study of radiation effects on tissues of the higher animals. Since the majority of the gross physiological and medical effects of radiation can apparently be explained on the cellular level, as involving chromatolysis and cell destruction in the more radiosensitive tissues, inhibition of cell multiplication, and chromosomal or mutagenic effects; and since tissue culture makes it possible to make direct observations on cell behavior, it is perhaps not surprising that radiation has been more thoroughly studied by means of tissue culture than any other toxic agent. It is the purpose of this paper to describe the current progress in this field as related to the medical and basic biological effects of radiation, to summarize our own recent observations on continuous radiation effects as exemplified in isotope therapy and toxicity, and to discuss the advantages and limitations of tissue culture as a means of quantitative study of such problems.

As early as 1914, about four years after tissue culture had been put on a firm basis, Price-Jones and Mottram cultivated mouse carcinoma cells in juxtaposition to seven milligrams of radium, and noted that while cell migration was not influenced by several hours of such radiation exposure, cell division was inhibited. It is of some interest to note the early date of this observation and the careful distinction made by these workers, since for many years after that time we note that some confusion existed in the field between the various criteria of growth in tissue culture, many observers using cell migration and growth almost interchangeably.

Those speakers in this symposium who have had much experience with tissue culture can appreciate Spear's account (1953) of the next significant investigation made by Strangeways and Oakeley (1923–1924). Oakeley, who was a visitor to Cambridge from South Africa in 1921, undertook to work on a

---

* From the Division of Biological and Medical Research, Argonne National Laboratory, Lemont, Illinois. Presented at the Eighth Annual Cancer Symposium, April, 1954, at the M. D. Anderson Hospital, Houston.

problem in tissue culture nine hours a day for six weeks before his return, and wound up by remaining in Cambridge for two years. These workers demonstrated that the effect on mitosis is not immediate, that recovery follows small doses, while larger doses are followed by abnormal mitosis, granular changes in the cytoplasm, and degeneration of cells.

The history of investigations on radiation effects in tissue culture has been dealt with at length in Spear's 1948 monograph. It is interesting that, while there has at no time been a widespread effort in this field of research, work has proceeded at a practically uniform, steady pace for 30 years. Interpretation of many of the earlier results in the light of modern concepts in quantitative radiobiology is hampered by the fact that radiation dosimetry was in a somewhat primitive state till the thirties; much of the more recent work, on the other hand, has tended to utilize tissue culture as a quantitative method in measuring biological effects of various types and qualities of radiation, while many of the more appropriate and unique virtues of tissue culture in studying the behavior of living cells, have been neglected. We shall not attempt to cover all of the accomplishments in this paper, but will rather discuss the present state of knowledge in the light of accumulated research, illustrating some of the points with our own material.

Immediate lethal effects on cultures are seen only after very high radiation doses have been administered. About 100,000 to 200,000 r appear to be required (Spear, 1931; Halberstaedter and Doljanski, 1937) to cause uniformly immediate death of cultures as indicated by their failure to show outgrowth after irradiation, although some cultures will fail to grow after doses of 25,000 r. Degeneration of some cells is seen, however, immediately after doses of 2500 to 10,000 r as well as later. This degeneration may take a number of forms (Lasnitski, 1943; Cox, 1931) including breakdown of the nuclear chromatin or fragmentation of the nucleus followed by cell death, or "explosive" dissolution of the cell.

Delayed death of cultures occurs after intermediate doses, which in the case of fibroblasts may be taken as the range between about 5000 and 100,000 r (Cox, 1931; Doljanski and Goldhaber, 1942; Lasnitski, 1943; Paterson, 1942). Delayed death has customarily been measured in terms of the length of time sub-

cultures may be carried before a subcultured explant fails to grow. As higher radiation doses are given within this range, the survival becomes shorter. As far as we know, no very definitive observations have been reported on the circumstances of this delayed death except those of Paterson (1942), who noted loss of the normal axial growth pattern of the cells and great cell enlargement. When these cultures recover, the recovery seems to be initiated by a few essentially undamaged cells in the culture.

At the lowest radiation doses, the predominant observable effects are on mitosis. These effects are readily detectable at 50 r, while after several hundred or a thousand r, recovery still takes place eventually. At the lowest doses where effects are perceptible the number of cells seen to be dividing becomes reduced and then, after a short period, returns to normal. After recovery the number may even exceed the normal value, an observation which has been taken to mean that while those cells about to enter mitosis have been delayed in doing so, others a little further back in the cycle are not so delayed, so that in effect two groups of cells (delayed and not delayed) reach the dividing stage together. It must be noted that many of the observations on which this is based have been made by counting the percentage of cells seen in mitosis at a given moment, so it is possible that this count could be increased, without a larger number of cells passing through mitosis, if a delay occurred during the stage of visible mitosis. We shall present evidence later that prolongation of visible mitosis is one of the effects of radiation, although we have not shown that it is necessarily a part of the recovery process.

When larger doses are given, mitosis may disappear altogether after an hour or so, while recovery occurs later. Generally speaking, recovery is more and more delayed with increasing dosage of radiation (Spear and Grimmett, 1933; Canti and Spear, 1927; Love, 1931; Lasnitski and Lea, 1940).

When a dose of radiation is given that prevents cells from entering mitosis, what apparently happens is that those cells which have already begun to divide—that is, reached prophase, continue to completion. It has been noted that almost all of these mitoses are complete by 80 minutes after irradiation; so it has been customary to do mitotic counts 80 minutes after a single irradiation and to consider that these counts reflect the full effect of the treatment(Spear and Grimmett, 1933). On this

basis it takes of the order of 50 r to reduce mitosis to one-half of the normal value (Lasnitski and Lea, 1940) while after 2000 r, it is about two days before cell division is seen again (Lasnitski, 1947). It might be well to note that mitosis normally proceeds in about one-half hour, so that 80 minutes would permit a considerably delayed mitosis to pass through.

In addition to inhibition of cell division, it has also been observed that mitoses are abnormal in appearance after irradiation. These abnormal mitoses may take a number of forms familiar to those who have studied radiation effects in various types of cells. Characteristically, one sees fragments of chromosomes which have been broken off and therefore do not follow the normal course during separation of the groups in anaphase; while sometimes two parts of chromosomes destined to go to the opposite poles have joined together before this, resulting in a bridge in anaphase which may prevent separation from taking place. This anomaly is so frequent, as well as so obvious, that it has been used as a measure of radiation dose in the range of a few hundred r. Another anomaly which has been much discussed is a "stickiness" of chromosomes in the various phases which impairs the mitotic process and is generally considered to be a physiological change in contrast to the structural ones just mentioned. Some observers (Koller, 1947) have also described impairment of the spindle mechanism, although others have failed to observe this. Cytochemical methods have also revealed a loss of nucleic acid from chromosomes in mitosis.

It is easy to see how these anomalies, as well as making the normal mitotic process more difficult to carry out, may result in loss or wrong distribution of chromatin and genic material in daughter cells, so that cells may eventually break down. Breakdown usually occurs at a later mitosis, and is no doubt an important factor in delayed damage (Lasnitski, 1943; Simon-Reuss and Spear, 1947).

Degeneration of cells, noted first in 1923 (Strangeways and Oakeley) has recently received increasing emphasis. This occurs in mitosis, and may occur fairly soon after irradiation or considerably later. The degree of degeneration is a function both of dose and of the mitotic activity of the tissue, and has been noted in the range between 100 and 10,000 r (Lasnitski, 1943). It is not necessarily characteristic of radiation alone, being seen after

various non-specific damaging agents (Kemp and Juul, 1931).

Certain types of cells are known to be much more radiosensitive than others, a fact which has great importance in relation to radiation therapy as well as the problem of radiation sickness. Probably a number of factors are involved here. While it must be pointed out that no single definition of radiosensitivity has been employed by those working with tissue cultures, workers have tended to define it in terms of the particular phenomenon they are observing, such as migration of cells or mitotic suppression—we shall assume that in discussing radiosensitivity here we are talking about loss of cells through degenerative changes. It is widely understood that in this sense, the most radiosensitive tissues are those having normally the most active mitosis; and this seems understandable in view of this discussion. There are certain exceptional cases, however, and the one that has attracted most attention is that of normal or malignant lymphocytes, whose radiosensitivity is well appreciated by radiologists. It has been suggested by Marshak (1942) that these cells may be sensitive to mitotic damage over a much longer stage in the "resting" period than is true of most cells; the sensitive stage has been taken as about three hours (Love, 1931) but judging by observations in lymphoma, it may be considerably longer. Trowell (1952) and Schrek (1948) have also shown that degeneration of resting lymphocytes in culture, apparently starting in the nucleus, follows quite low radiation doses, which most types of resting cells survive without any visible evidence of damage.

One recent study of radiation effects on cell growth deserves particular mention, since it represents the first attempt to use strictly quantitative methods. Reid and Gifford (1952), using Earle's methods and cultivating strain L mouse fibroblasts, followed the growth of these cells after doses of 250 to 2000 r. All of these doses of X irradiation caused a slowing of the cell multiplication rate. After 1000 and 2000 r, the number of cells slowly decreased, while 500 r caused a slowing of growth, a delayed destruction of about half of the surviving cells at about two weeks, and eventual recovery (probably of a few normal survivors) to normal growth rate in the third week. 250 r gave only a transient lag in growth.

Tissue culture has been widely employed, alongside of tissue *in vivo*, to get at the question of the indirect effects of radiation due to the effects of radiation on other tissue or on the vascular bed in the living animal. For example, while a transplantable mouse carcinoma irradiated in the animal grows well in tissue culture if explanted at once, a few days' residence in the animal makes it incapable of cultivation, suggesting that something like damage to the vascular bed of the host is a factor in tumor regression (Simon-Reuss and Spear, 1947).

Malignant cells have not shown any very striking differences in radiosensitivity in tissue culture as compared with homologous normal cells. We have already mentioned the radiosensitivity of normal and malignant lymphocytes. Furth and co-workers (1938) made a quantitative study of lymphoma cells irradiated *in vitro*, estimating their survival by transplantability. According to their figures, one million irradiated cells, given 1000 r, were nearly killed; the transplantability of this inoculum corresponded to that of a single unirradiated cell. A sarcoma, on the other hand, was hardly altered by 2000 r. Doljanski and Goldhaber (1944) found that the delayed lethal dose of X-rays for rat sarcoma cells was around 2000 r, while that for normal fibroblasts of the chicken was 5000 r. Goldfeder (1949), who has made detailed studies of the radiosensitivities of two mouse carcinomas observes that one of these is more sensitive *in vivo*, and the other *in vitro*.

Recent understanding of the marked effect of oxygen on the radiosensitivity of many biological objects necessitates a re-evaluation of many experiments in which radiosensitivity of tissues under different physiological conditions has been measured, and may serve to explain some of the discrepancies that have been noted. Hall (1952) in a study of the radiosensitivity of tumor fragments, found that such conditions as the size of the fragments and the temperature at which irradiation was done, strikingly altered their responses to X-ray, owing to the influence of these conditions on oxygen concentration in the irradiated fragments.

Our own studies in this field have been devoted to investigating the effects of continuous irradiation on cultures. Work of this sort has been difficult to carry out; this is certainly true when one has

to rely on the X-ray machine. We have used two different techniques. One is the implantation of point sources of beta radiation, which are glass beads containing carrier free radioactive strontium or yttrium (Kisieleski, *et al.*, 1950). The usefulness of this method is that the dose rate drops off very rapidly over a distance of a few millimeters from the source, so that we can observe simultaneously the effects on cells of both large and small doses. It has the disadvantage that it is practically impossible to compute the dose rate at any point with any degree of accuracy. Our other method has been incorporation of tritium, as the oxide, into the water of the medium. This hydrogen isotope yields an extremely soft beta ray with a range less than the thickness of a coverslip, so that a culture can be studied while it is being heavily irradiated without hazard to the observer; also, being interchangeable with the hydrogen in water, one is assured of a practically uniform dosage throughout the culture. Our material consists of cultures of chick embryo muscle irradiated by these two techniques.

The first problem that attracted our attention was the nature of the prolongation of mitosis which occurs as a result of irradiation. Studies with point sources in tissue (Brues and Rietz, 1951) had shown that after 24 to 48 hours' irradiation at high dose rates (above 1 or 2 r per minute) mitosis practically ceased, while at lower dose rates it proceeded at a normal rate. A few mitoses were always observed, however, in the heavily irradiated area, and it was thought that they might be delayed mitoses, although morphologically they seemed essentially normal. It was also thought worthwhile to make a careful study of mitotic behavior by the use of motion pictures (Brues and Stroud, 1950; Brues, *et al.*, 1951).

It was clear from cinematographic records that under irradiation intensities on the order of one r per minute, most of the mitoses took longer than normal to go to completion, yet that a few figures were found which proceeded in an entirely normal fashion. Because of the difficulty of finding a cell just about to enter prophase, however, the duration of mitotic delay could not be made out accurately in this way, so we resorted to the laborious method of making serial photographs, every five minutes, of areas of cultures containing about one hundred cells.

We have found, by this method, that the mean duration of mitosis is about doubled at these doses, but that there is great variation in the length of the delay, some passing through in normal time, others taking over two hours. It was also noted that while the rate of cells entering mitosis was very little affected during the first hour, the delay during mitosis resulted in a higher concentration of mitotic cells than normal; if one were to judge the mitotic rate by the mitotic index, one would have concluded that there was actually a stimulation of cell division. It is interesting to recall that colchicine was originally presumed to be a stimulator of cell division on such a basis as this. Accordingly, reports of stimulating effects of radiation based on the mitotic index must be accepted with caution.

Three stages of cell impairment are observed at widely different levels of dose rate, and they correspond to those that have been described after single doses of radiation. At the lowest dose

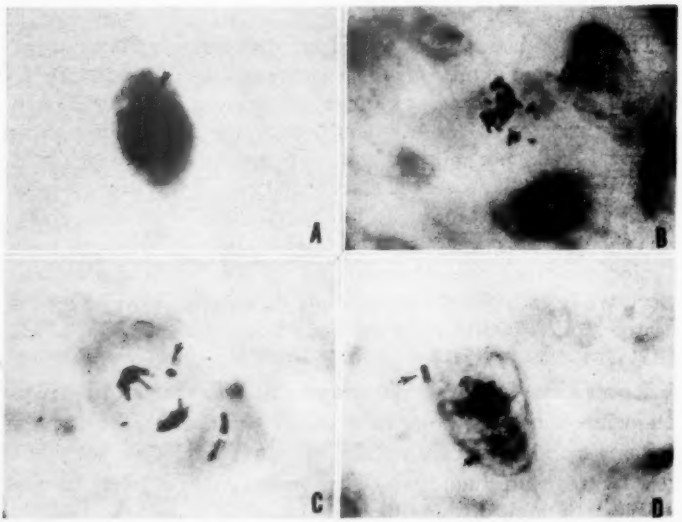


PLATE I. Feulgen stained fibroblast cells in tissue culture showing the effects of radiation by Tritium (H^3). A. Nuclear division without cytoplasmic division with the result of lost and clumped chromosomes; 2,000 rep. B. Displaced chromosomes at early anaphase; 2,000 rep. C. Broken and lost chromosomes at late anaphase; 5,000 rep. D. Unattached chromosomes at metaphase; 5,000 rep. (A and B at 67 rep/hour; C and D at 167 rep/hour.

rates (40 to 200 r per hour) we see the familiar evidences of chromosome damage, some leading to death of the cells, illustrated in the accompanying photographs of stained material (Plates I–III). In the cinematographs, further anomalies in cell behavior were noted and some of these are illustrated shortly by drawings traced from cinematographs (Fig. 1)[1]. It was noted in particular, that the various accompaniments of the mitotic process appeared to be out of phase with one another. Normally, just at the beginning of anaphase, the cell, which has rounded up during prophase, begins to undergo an intense surface motion while the cleavage furrow develops. This motion has been intensively studied by Lettré (1952), who has evidence to indicate that it is related to energy metabolism in the cytoplasm, perhaps related to formation of the spindle gel. We have noted that in irradiated cells, this motion may commence before anaphase

[1] Ed. This could be "Plate IV."

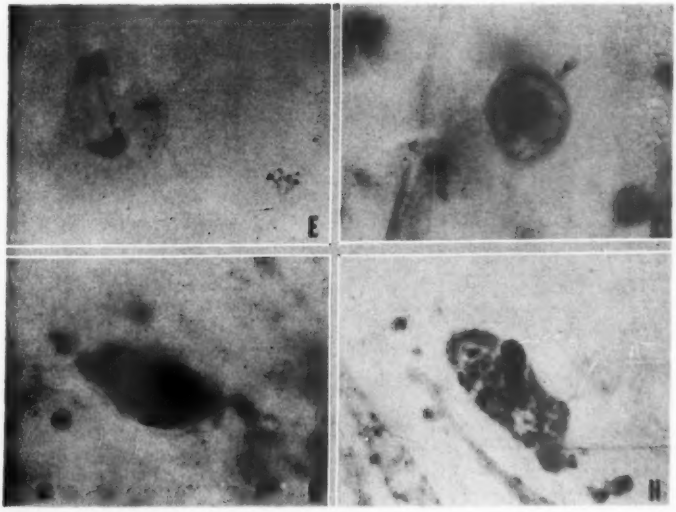


PLATE II. Feulgen stained fibroblast cells in tissue culture showing the effects of radiation by tritium (H^3). E. Lagging chromosome with chromatin substance at anaphase; 8,700 rep. F. Clumped chromosomes and chromosome intrachange at metaphase; 8,700 rep. G. Disorganized and misplaced chromosomes at anaphase with apparent spindle destruction; 8,700 rep. H. Degenerate cell with chromatin massed in globules; 8,700 rep. (All at 167 rep/hour.)

begins, or that it may be sluggish or absent throughout the cycle. It may also be limited to a part of the cell (Fig. 1d). Again, the cleavage furrow may fail to develop, or may retract after starting to form. In some cases, the position of the metaphase plate indicates that it is not held in position with the normal consistency; it may move back and forth in the cell, as if trying to find the cleavage furrow (Fig. 1a), or the whole figure may rotate as a gel freed of the normal restrictions of the cytoplasm; a phenomenon similar to rotation of the whole nucleus in the resting cell described by Pomerat (1953). Triasters have also been observed (Fig. 1c). All of these things lead one to suspect that the spindle and gelation mechanisms have been impaired, and this impairment can take a large number of forms. It would be tempting to think that this might be due to direct effects on the spindle fibers; but this seems unlikely in view of Zirkle and Bloom's observation (1953) that isolated irradiation of the spindle by a proton beam has no effect on mitosis. It is there-

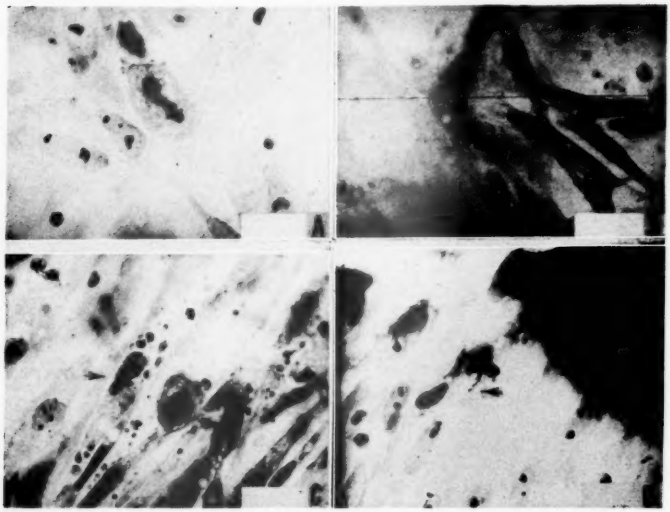


PLATE III. Feulgen stained fibroblast cells in tissue culture showing the effects of radiation by radioactive Yttrium (Y^{91}) beads. Doses are on the order of 250 rep/hour. In the case of D, the dark area is the bead and the dose rate is 1,000–2,000 rep/hour. A. Chromosome loss. B. Chromosome bridge. C. Clumped chromosomes in metaphase and darkly staining granules. D. Broken chromosomes.

fore more likely that this represents a physiological change involving the whole cell.

Effects of larger doses are also shown in the photographs. After two days' irradiation at a rate of 200 to 400 r per hour, it is noted that the resting cells have developed densely staining granules in the cytoplasm (Plates IIH and IIIC), although most of these cells still appear intact. On the other hand, in this dosage range cells still remain somewhat active, and will migrate. This is seen in plate IV, where migration of cells from an explant attached to a radioactive bead is seen to take place. At still higher dose rates, large vacuoles appear, and we have observed ex-

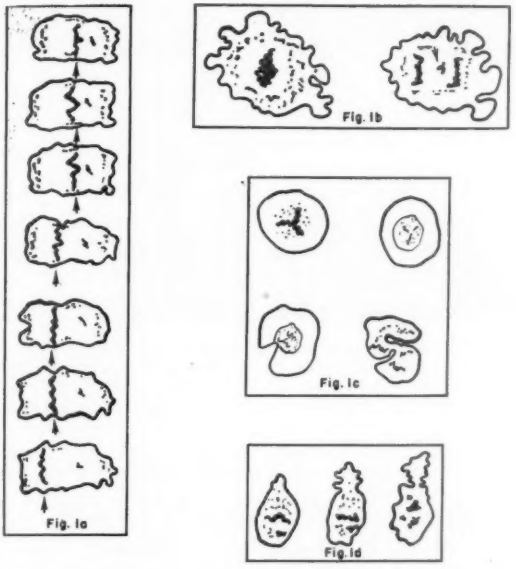


FIG. I. Drawings traced from motion picture films of fibroblasts grown in H^3_2O solution, showing mitotic anomalies associated with delayed division.

a. Metaphase plate shifting position rapidly in the cell; separation at equatorial plate inhibited; abortive attempts at cleavage. This cell entered anaphase after about an hour. Frames 10 seconds apart.

b. Metaphase going into anaphase with unattached chromosomes or fragments. Frames 3 minutes apart.

c. Triaster with attempt to form 3 daughter cells after which the chromosome group becomes indistinct; note that a single furrow forms, followed by a second furrow at right angles to it. Two hours later this had coalesced into a single cell.

d. Delayed metaphase (4 hours) with clumping of mitochondria. Attempt at cleavage with surface activity, limited to area distal to the chromosome mass on the right.

plosive destruction occurring. This process takes less than a minute; the cell surface suddenly expands to about twice the previous diameter of the cell, and immediately afterward the nucleus shrinks, discharging a clear nuclear sap.

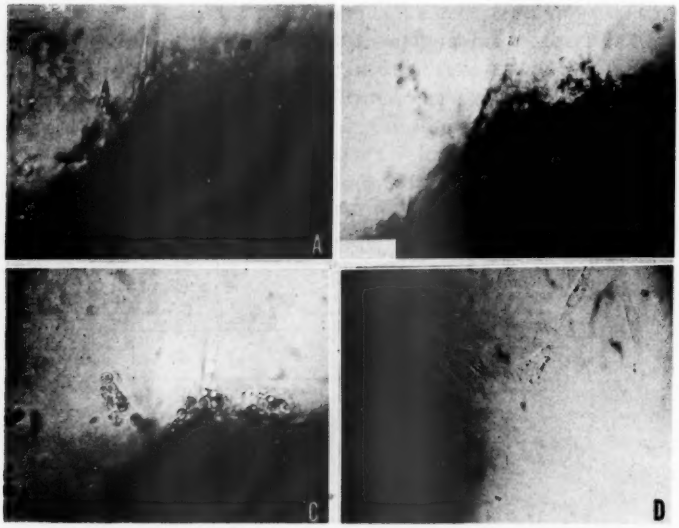


PLATE IV. Embryonic chick heart tissue and cells adhering to radioactive Yttrium (Y^{90}) bead in tissue culture. A. 27 hours incubation with the bead at a dose rate estimated at 1,000–2,000 rep/hour. B. 30 hours later. C. 79 hours later. D. Another area with pinocytotic cells from an adjacent explant, 79 hours incubation.

Summary

The literature of radiation effects in tissue cultures has been reviewed. Visible effects occur over a wide range of dosages; at lower dosages (in the order of 100–1000 rep) inhibition of mitosis and chromosome damage are predominant. To kill cells immediately requires very high dosages, on the order of 100,000 rep. Intermediate dosages result in delayed death of cells.

Three types of damage leading to cell destruction can be identified in ascending order of dosage: (1) death of cells as a result of chromosome or other mitotic damage incurred at mitosis, manifested at once or at a later mitosis; (2) delayed death of

cells where injury was apparently incurred in the resting stage but appeared at a time when the cell was about to enter mitosis; and (3) "explosions" of resting cells with immediate nuclear shrinkage.

Our own observations, made under conditions of continuous radiation dosage, have confirmed these earlier findings. In addition, a predominant feature of the process is a prolongation of mitosis associated with a variety of anomalies. These anomalies, which are described in detail, seem to be related to alterations in the normal physiological accompaniment of mitosis (gelation, spindle fixation, surface activity, and cleavage). It is pointed out that the delay of mitosis renders inaccurate the measurement of proliferation rate by estimating the mitotic index.

REFERENCES

Brues, A. M., and A. N. Stroud: *Anat. Rec.*, 106: 181, 1950.

Brues, A. M., A. N. Stroud, and G. Svihla: *Anat. Rec.*, 109: 411, 1951.

Brues, A. M., and L. Rietz: *Ann. New York Acad. Sci.*, 51: 1497, 1951.

Canti, R. G., and F. G. Spear: *Proc. Roy. Soc. B.*, 102: 92, 1927.

Cox, S. F.: *Arch. Exp. Zellforsch.*, 11: 122, 1931.

Doljanski, L., and G. Goldhaber: *Growth*, 6: 235, 1942.

Doljanski, L., and G. Goldhaber: *Cancer Research*, 4: 106, 1944.

Furth, J., A. Tuggle, and C. Breedis: *Proc. Soc. Exp. Biol. and Med.*, 38: 490, 1938.

Golfeder, A.: *Radiol.*, 52: 230, 1949.

Halberstaedter, L., and L. Doljanski: *Arch. Exp. Zellforsch.*, 19: 475, 1937.

Hall, B. V., K. Hamilton, and A. M. Brues: *Cancer Research*, 12: 268, 1952.

Kemp, T., and J. Juul: *Arch. Exp. Zellforsch.*, 11: 602, 1931.

Kisieleski, W. E., G. Svihla, and A. M. Brues: *Science*, 112: 400, 1950.

Koller, P. C.: *Brit. J. Radiol.*, Suppl. 1: 85, 1947.

Lasnitzki, I., and D. E. Lea: *Brit. J. Radiol.*, 13: 149, 1940.

Lasnitzki, I.: *Brit. J. Radiol.*, 20: 240, 1947.

Lasnitzki, I.: *Brit. J. Radiol.*, 16: 137, 1943.

Lettré, H.: *Cancer Research*, 12: 847, 1952.

Love, W. H.: *Arch. Exp. Zellforsch.*, 11: 435, 1931.

Marshak, A.: *Radiol.*, 39: 621, 1942.

Paterson, E.: *Brit. J. Radiol.*, 15: 257, 1942.

Price-Jones, C., and J. C. Mottram: *London, Middlesex Hospital Arch.*, 33: 21, 1914.

Pomerat, C. M.: Personal communications, 1953.

Reid, T. R., and M. P. Gifford: *J. Nat. Cancer Inst.*, 13: 431, 1952.

Schrek, R.: *Am. J. Path.*, 24: 1055, 1948.

Simon-Reuss, I., and F. G. Spear: *Brit. J. Radiol.*, 20: 63, 1947.

Spear, F. G.: *Proc. Roy. Soc. B.*, 108: 190, 1931.

Spear, F. G., and L. G. Grimmett: *Brit. J. Radiol.*, 6: 387, 1933.

Spear, F. G.: *Brit. J. Radiol.*, 8: 68, 1935.

Spear, F. G.: *Tissue Culture and Experimental Radiology*. Bristol, John Wright and Sons, Ltd., 1948.

Strangeways, T. S. P., and H. E. H. Oakeley: *Proc. Roy. Soc. B.*, 95: 373, 1923–1924.

Trowell, O. A.: *J. Path. Bact.*, 64: 687, 1952.

Zirkle, R. E., and W. Bloom: *Science*, 117: 487, 1953.

DISTRIBUTION OF RADIOIODINATED ANTI-ORGAN ANTIBODIES IN THE INJECTED RAT*

LUDWIK ANIGSTEIN, KENNETH P. MCCONNELL,† DOROTHY M. WHITNEY, PETER PAPPAS,‡ OSCAR W. PORTMAN,§ AND WILLIAM BARNES||

The history of anti-organ serum began more than fifty years ago when Metchnikoff (1900) produced a serum from guinea pigs inoculated with rabbit testicular tissue. This serum proved spermotoxic to rabbits. At the same time Bordet (1900) prepared a hemolytic serum against erythrocytes. These epoch making observations were followed by extensive studies on serological properties of various animal tissues leading to the concept of species and organ specificity (Landsteiner, 1945).

Regardless of the emphasis placed upon anti-tissue serum as a cytotoxic factor (Metchnikoff, 1900) or as a cellular stimulating agent (Bogomolets, 1943), the mechanism involved depends largely upon the affinity of the antibody for the tissue against which it has been prepared. More impressive, however, is the damaging influence of the antiserum by its functional and pathological effects. These could be demonstrated and interpreted as resulting from antigen-antibody reaction *in vivo*. In this respect the anti-kidney serum prepared by Smadel (1936) in immunizing rabbits with rat kidney tissue induced a drastic nephrotoxic effect when injected into rats. Functional and structural renal lesions were produced in rats by injection of nephrotoxic globulin as reported by Lippman, Marti, and Campbell (1952). Hence, the activity of the anti-organ serum *in vivo* may result in characteristic pathological lesions such as nephritis, apparently due to localization in the tissue of organ specific antibodies.

---

* From the Department of Preventive Medicine and Public Health and the Department of Biochemistry, The University of Texas Medical Branch, Galveston. This work was supported by a Contract (NR: 130–981) between the Office of Naval Research, Department of the Navy, and the University of Texas. Received for publication August 16, 1954.

† Veterans Administration Hospital, Louisville, Ky.
‡ William Beaumont Army Hospital, El Paso, Texas.
§ University of California, Department of Physiology, Berkeley, Calif.
|| Fitzsimons Army Hospital, Denver, Colo.

A suppression of the defense mechanism of the spleen by the reticulo-endothelial immune serum (REIS) was demonstrated by anemia and bartonellosis of bartonella carrier rats injected with an antiserum prepared against rat spleen and bone marrow (Anigstein and Pomerat, 1945). The damaging factor of this antiserum was found to be in the serum globulin fraction (Frieden, Pomerat, and Anigstein, 1945). Furthermore, a very potent anemia-inducing factor was found by Pomerat, Frieden, and Yeager (1947) in the anti-blood serum globulins. These few examples indicate that actually it was the cytotoxic, damaging effect of the tissue antiserum which served as criterion of the specific activity of the antibody *in vivo*. However, with the new developments in the tracer chemistry techniques (dyes, fluorescence) and with the advent of radioisotopes tracers in particular, it was shown that by coupling of the antibody molecule with traceable chemical groupings, the distribution and the localization of the antibody *in vivo* can actually be revealed.

In this regard, a new chapter in immunochemistry has been developed by Pressman and Keighley (1948) in labelling the antibody molecule with radioactive iodine. This method proved a most valuable tool for tracing the distribution and localization of antibodies in various tissues of the injected host (Anigstein, 1953). For example, radioiodinated anti-rat-kidney serum, injected into rats, was found mainly localized in the kidney glomeruli although some radioactivity was taken up by the liver, lung and spleen (Pressman and Keighley, 1948). It was further shown that anti-rat-lung serum contained antibody which localized specifically in the lung, but also cross-reacted with the kidney (Pressman and Eisen, 1950). These cross-reactions are probably due to common antigenic components of the various tissues, particularly to those present in the vascular bed (Pressman and Sherman, 1951). In fact, anti-rat-aorta antibodies were found in common with the vascular beds of liver, kidney and lung of the rat (Pressman, *et al.*, 1952).

The objectives of the present study are the distribution of the radioiodinated globulin fraction of the anti-organ rabbit sera in the rat as the recipient animal host, and the evaluation of the radioactivity concentration carried by the specific antibody to the homologous organ as ultimate destination.

An attempt was made in the present inquiry to explore some activities and properties of the anti-rat-liver serum and of anti-rat-pancreas serum.

Although the extensive serological analysis of cell antigens has included in the past almost all tissue components of the animal body, the study of the antigenic properties of the pancreas apparently has been neglected. In fact, neither a successful anti-pancreas serum nor its action have ever been reported. The first recorded attempt to use pancreas as tissue antigen was made by Besredka (1900) who injected rabbit pancreas tissue into a guinea pig. The resulting antiserum showed cytotoxic effect upon rabbit's leukocytes. Furthermore, Witebsky (1929) briefly mentions the organ specificity of the pancreas antiserum. Finally, Henle, Chambers, and Groupe (1941) comment that the only antisera that did not show any serological reactivity were those against murine pancreas suspensions.

Material and Methods

Preparation of anti-organ sera. Anti-organ sera against rat tissues were prepared by repeated injections of male rabbits weighing about 3 kg. with suspensions of perfused rat organs, namely kidney, liver, spleen, lung, and pancreas. In accordance with previous authors (Smadel, 1936; Pressman and Keighley, 1948) considerable attention was paid to the perfusion of rats in order to preserve specific antigenicity of tissues used for immunization of rabbits, as well as to avoid such undesirable side-effects as hematuria or hemoglobinuria (Lippman, *et al.*, 1952) induced by anti-kidney serum if unwashed kidney is used (Smadel, 1936). Before sacrificing, rats were injected intra-abdominally with Nembutal-sodium (5–6 mg. per rat) with a heparinized syringe, and then perfused with 1–2 liters of saline (0.9 per cent) passing by gravity through a canula inserted into the adbominal aorta. The perfusate was allowed to leave by incision in the vena cava until the tissues appeared bloodless. Usually, the bleaching of the kidney, liver, and lung was accomplished within 30–60 minutes, whereas the appearance of the spleen was hardly affected even by longer perfusion. The bloodless organs were then removed, minced, homogenized and 20 per cent of the homogenate suspension in saline centrifuged for

5 minutes at 1500 rpm. Then the supernate (0.25 to 0.75 ml.) was injected intravenously into rabbits twice a week over a period of 3–4 weeks. At the same time, rabbits were injected with the tissue centrifugate intra-abdominally (5.0 ml. of 20 per cent suspension). In the preparation of the anti-pancreas serum the homogenate of two rat pancreas was injected intra-abdominally into rabbits in addition to the intravenously injected supernate.

Rabbits were bled 8 days after the last injection. The titers of the sera were determined by the precipitin test with fresh tissue antigen. The precipitin test was found more reliable than the complement fixation technique, particularly for the anti-pancreas serum because of the hemolytic property of the fresh pancreas antigen. Precipitin titers from 1:100 up to 1:10,000 were obtained. In addition to the antigenic peculiarity of the pancreas, its anatomical features deserve attention with reference to the diffuse and dendritic character of the rat organ "extending from the end of the duodenal fold to the left into the gastro-splenic omentum, where it ramifies, the chief part of the duodenal pancreas following the curve of the gut, but ramifying in its wide mesentery . . ." (Greene, 1935). The ramifications into the omentum render the strict separation of the pancreas difficult. However, the pinkish color and the sinking of the pancreas when placed in a beaker with iced water, in contrast to the white omentum which floats, are helpful criteria. The unavoidable admixture of the fatty omentum in some of the pancreas material used for immunization could hardly interfere with the antibody response of the rabbit.

A continuous supply of high titer antisera was secured by giving rabbits a single "booster shot" with the same tissue antigen 30 days after completion of immunization.

Fractionation of antisera. In all experiments only the globulin fractions of the sera were used for labelling with I^{131} and for rat inoculation. The ammonium sulfate precipitation in cellophane tubing (Eisen and Pressman, 1950), was used in a dialysing system in concentration of 1.75 M. ammonium sulfate. This was rocked overnight in refrigerator and was followed by rotary dialysis at about 5 C. against 17 liters of 0.16 M. saline until the dialysate was ammonium sulfate free. The precipitated globulin fractions were then lyophilized.

Radioiodination. The serum globulin was radioiodinated by special arrangement with Dr. D. L. Tabern of Abbott Laboratories and carried out by G. I. Gleason of the Department of Radioactive Pharmaceuticals, Oak Ridge and Chicago. Iodine from 50 micrograms of potassium iodine was incorporated into 100 milligrams of globulin, corresponding to approximately one atom of iodine per molecule of protein. This low iodination ratio is well within the stability limits for radiobound protein without destroying its serological specificity (Pressman and Sternberger, 1950). The specific activity of the radioactive material was on the average from 100 to 200 microcuries/cc. with an average globulin concentration of 2–4 mg./cc. Male rats, approximately 250 gram weight, (Sprague-Dawley strain) were injected with radioiodinated gamma globulin solution. Intracardial injections of rats used throughout the experiments provided a uniform and rapid distribution of the material. Rats were sacrificed in groups of three or four over a period of 14–19 days, following an intra-abdominal injection of Nembutal-sodium. Blood samples (0.2cc.) were drawn from the heart of rats at various intervals after injection with radioactive globulin.

Radioactivity measurements. Duplicate samples of the various tissues for measurements of radioactivity were taken in amounts of 0.2 to 0.4 g. The tissues were minced, weighed in bottle caps and digested in 1.0 ml. of N NaOH added to each cap. The digestion of tissues or blood proceeded overnight in 37° C. or in room temperature. The tissue or blood samples were assayed for radioactivity with the 64 Scaler Tracerlab counter employing a thin window Geiger-Muller tube. Corrections for radioactive decay and for dosage based on 250 g. standard rat were made. The counts per minute per gram of wet perfused tissues were then plotted against time on semilogarithmic paper. The biological half-life is determined from the slope of the curve thus obtained. These values represent indices of the relative rates of elimination of the radioactive carrier from the homologous and heterologous tissues.

Experiments and Results

Distribution of the anti-rat-liver radioactivity carrier. The preparations of the anti-rat-liver serum was originally reported

by Pressman and Eisen (1950), but no specific localization of the antibodies was observed in the rat liver. However the failure was not considered by the authors as conclusive and it was believed that antisera prepared differently may lead to localization. When rat liver blood vessels were used for immunization of rabbits, antibodies against liver, kidney, and lung could be demonstrated *in vivo* (Pressman, *et al.*, 1951).

In experimenting with various techniques of anti-organ sera production, the double route of immunization, namely the intravenous injection of the supernate and the intra-abdominal inoculation of the tissue residuum was chosen by us as previously described. It was thought that this procedure will induce a more complete antibody response to the manifold antigenic components of the tissue.

The single intracardial injection of radioiodinated globulin solution into rats provided an immediate mixing in the circulating blood and uniform distribution of the material. Nevertheless, with the corrections to standard weight of the rats, the blood levels varied from animal to animal in intensity and duration of radioactivity. These variations are influenced not only by the specific activity of the injected radio-bound protein but by the rate of elimination from the circulating blood.

The amount of radioactivity found in the blood was throughout the observation periods in various series of rats always several times higher than in the tissues. On the average, the values in the tissues were approximately 1/10 to 1/30 of corresponding blood values as compared with 1/4 to 1/20 in the experience of Latta (1951) whose experimental animals were not perfused. Since our animals were routinely perfused as completely as possible, we have reason to believe that most or all recorded radioactivity is due to specific activity of the tissue, regardless of the vascularity of the organ.

Radioactivity concentrations in tissues can be determined by the tissue to blood (T/B) ratio obtained by dividing counts for various tissues by the values for blood of the same rat (Eisen and Pressman, 1950). This index proved valuable for the degree of retention of radioactivity by the tissues relatively to the elimination rates from the blood, particularly during the first days after injection. However for the relative radioactivity, values for the homologous tissue as compared with the heterol-

ogous, the tissue to tissue ratio was found more indicative. This index (T/T), first introduced by the authors in the present study is obtained by dividing counts per minute per gram of the homologous organ by the counts of the heterologous tissue.

Preliminary tests for the distribution of radioactivity in rat tissues were carried out on 17 rats of which 8 were injected with radioiodinated anti-rat-liver globulin solution containing about 300 microcuries per 1.00 cc. (at the time of the initial assay). In addition, 9 rats were injected with similarly tagged normal rabbit globulin. Samples of blood, liver, kidney, lung, and spleen and pancreas were prepared in duplicate from both series of rats 3 and 7 days after injection and measured for radioactivity. Although cross-reactions were found in all the examined tissues, the liver of the "anti-liver" rat series showed higher T/T values for lung, kidney, spleen, and pancreas as compared with corresponding values of the "normal" globulin series of rats. For example, the radioactivity concentration of the liver exceeds that of the lung in the average almost 3 times and the radioactivity of the pancreas as much as 8 times (Table 1). The higher values

TABLE 1

Radioactivity expressed in liver to tissue ratios (T/T) of 8 rats injected with I 131 anti-rat-liver rabbit gamma globulin as compared with ratios of 9 rats injected with I 131 normal rabbit serum globulin

Rat No.	Time after Injection	Li/Bl	Li/Lu	Li/Ki	Li/Sp	Li/Pa
	Days					
1	3	0.65	1.50	2.01	1.61	12.0
2	3	0.80	2.98	1.54	1.70	3.62
3	3	1.21	3.10	2.00	1.96	9.00
4	3	0.35	1.79	0.46	1.29	4.42
5	6	1.17	5.40	1.14	1.75	7.53
6	6	1.35	3.50	1.32	2.55	5.20
7	7	1.02	3.06	1.65	0.77	20.50
8	7	0.69	2.00	0.73	0.72	2.82
Average		0.90	2.92	1.36	1.84	8.12
T/T averages of 9 "normal" rats		0.12	0.66	0.48	0.59	0.97

for the liver as a homologous tissue in this series of experiments as compared with the lower radioactivity in the heterologous organs speak for a relative accumulation in the liver. In other

words, the radioactive carrier is picked up by preferential affinity by the homologous liver tissue.

Similar experiments were conducted on several groups of a series of 39 rats. Of these, 24 were injected with I^{131} anti-rat-liver rabbit globulin, whereas 15 rats injected with "normal" rabbit globulin served as controls. The distribution of radioactivity in the blood and among organs of 24 rats injected with anti-rat-liver globulin was followed from the 2nd to 17th day after injection at 3 day intervals (Table 2). The values for blood and tissues were calculated as an average from duplicate samples of 4 rats in each group and corrected to day of perfusion. There was a rapid decrease of radioactivity in the blood and tissues during the first week after injection. The decrease was particularly marked in the pancreas with an activity on the fifth day of 1/30 of that in the blood. This was followed by a steady decline of subsequent values for other tissues. The declining radioactivity in the liver almost approached the blood values at the end of the observation period. Widely spread cross-reactions of heterologous tissues to anti-liver antibodies were recorded and can probably be explained by the antigenic similarity of liver, kidney, and lung as interpreted by Pressman and Sherman (1951). This common antigenicity may also be a factor which perhaps may partly divert the specific antibodies before their fixation with the homologous organ. While the total counts do not reflect the absolute concentration of radioactivity in the liver, the T/T ratios indicate a relative accumulation as compared with heterologous tissues (Table 2). This applies in particular to the liver to pancreas ratios which indicate values for the liver consistently higher, up to seven times that of the pancreas at the end of the observation period. Relatively high are also the ratios of the liver to lung and to kidney (Table 2). To the contrary, the series of rats injected with "normal" rabbit globulin show considerably lower T/T ratios although the rapid decline in radioactivity of the pancreas shows an apparent accumulation in the liver (Table 3). The duration of radioactivity in various tissues was calculated in terms of biological half-life data. For this purpose the recorded concentration of radioactivity per gram of wet tissue was plotted logarithmically (on the basis of the initial injection) against time (Fig. I). From the slopes of the straight curves the biological half-life of radioactivity was

determined. Corrections have been made for decay of I^{131} from time of perfusion to the time of counting.

TABLE 2

Distribution of radioactivity in tissues of 24 rats injected with radioiodinated anti-rat-liver rabbit globulin (1.6 mg. globulin and 224 microcuries per rat)

Rat Nos.	Days after Injection	Radioactivity values (cpm/g) in tissues*					Tissue/Tissue ratio			
		Blood	Liver	Kidney	Lung	Pancreas	Li/Bl	Li/Ki	Li/Lu	Li/Pa
5, 6, 7, 9....	2	314,937	39,280	43,019	51,001	29,799	0.12	0.91	0.75	1.32
8, 10, 15, 16	5	146,211	20,561	17,660	34,959	5,693	0.14	1.16	0.59	3.61
17, 18, 25, 26	8	58,208	13,705	14,954	15,815	2,567	0.24	0.92	0.87	5.34
23, 24, 33, 34	11	32,570	8,350	7,417	6,010	1,652	0.26	1.12	1.39	5.05
31, 32, 41, 42	14	11,148	6,187	7,408	3,391	1,638	0.55	0.84	1.82	3.87
39, 47, 48, 49	17	5,985	4,148	4,059	2,548	590	0.69	1.02	1.63	7.03

* Average counts of duplicate blood and tissue samples from groups of 4 rats. Corrected for decay to day of injection.

Data obtained for rats injected with anti-rat-liver globulin when compared with values obtained for rats treated with "normal" rabbit globulin as control in the present study showed identical half-life for blood (2.4 days), otherwise the half life for the other tissues was longer than in the controls:

Rat tissue	Normal rabbit globulin injected	Anti-rat-liver globulin injected
	Days	Days
Blood	2.4	2.4
Liver	3.7	4.9
Pancreas	2.4	3.0
Lung	2.5	2.7
Kidney	3.9	5.8

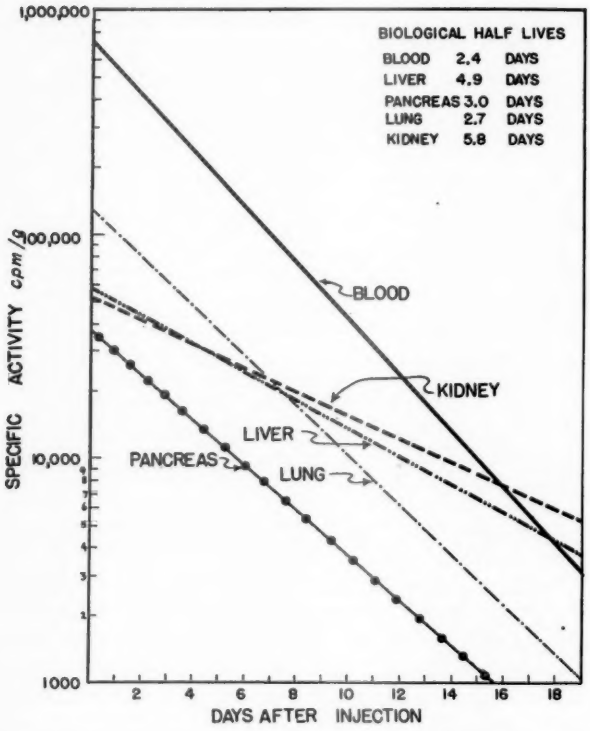
TABLE 3

Distribution of radioactivity in tissues of 15 rats injected with radioiodinated normal rabbit globulin (0.8 mg. and 40 microcuries per rat)

Rat Nos.	Days after Injection	Radioactivity values (cpm/g) in tissues*					Tissue/Tissue ratio			
		Blood	Liver	Kidney	Lung	Pancreas	Li/Bl	Li/Ki	Li/Lu	Li/Pa
50, 51, 52...	2	54,770	4,452	9,072	11,433	8,123	0.08	0.50	0.39	0.55
53, 54, 55...	5	23,387	2,898	4,852	4,183	2,393	0.12	0.50	0.70	1.21
56, 57, 58...	8	15,643	1,606	3,309	4,053	1,865	0.10	0.47	0.40	0.87
59, 60, 61...11		4,660	747	1,662	978	570	0.16	0.44	0.76	1.31
62, 63, 64...13		2,303	612	1,351	997	397	0.26	0.45	0.61	1.54

* Average counts of blood and tissue samples from groups of 3 rats. Counts corrected for decay to day of injection.

FIG. 1 BIOLOGICAL DECAY CURVES PLOTTED LOGARITHMICALLY SHOWING RADIOACTIVITY ELIMINATION FROM TISSUES OF RATS INJECTED WITH I^{131} ANTI-RAT-LIVER RABBIT SERUM GLOBULIN.



Distribution of an anti-rat-pancreas radioactivity carrier. Several lots of anti-rat-pancreas sera were made available by various immunization procedures, but similar to the anti-liver serum, the intra-abdominal and intravenous routes of antigen administration was chosen with the idea to obtain an optimum

In general, the radioactivity concentration in the pancreas of rats injected with I 131 anti-pancreas globulin was found within antibody response. The precipitin test titers with fresh pancreas antigen ranged from 1:100 to 10,000.

the levels of other tissues (Table 4), but beginning with the 8th day the elimination rate from the pancreas was apparently increased. Some of the counts were even too low for validity of calculation. These observations covering 14 days period were based on a series of 15 rats injected with 0.75 mg. globulin and 30 microcuries each. Duplicate samples of blood, liver, kidney, lung, pancreas, and spleen were measured for radioactivity and biological decay curves plotted against time. From the linear type of the plots the biological half-life for the tissues was determined as follows:

	Days		Days
Blood	2.4	Lung	3.7
Liver	2.8	Kidney	3.5
Pancreas	3.0	Spleen	3.9

TABLE 4

Distribution of Radioactivity in Tissues of 15 Rats Injected with I^{131} Anti-Rat-Pancreas Serum Globulin (0.75 mg. and 30 microcuries per rat)

Days after Injection	Radioactivity in Tissues (cpm/g)					
	Blood	Liver	Kidney	Lung	Pancreas	Spleen
2	36,600	4,280	6,155	7,250	5,300	7,430
	27,500	5,190	8,080	25,400	5,380	8,410
	28,000	4,480	6,320	8,510	2,780	6,290
5	9,015	2,980	4,960	4,490	1,670	5,600
	5,950	950	1,925	2,460	1,220	1,940
	12,300	2,170	4,190	4,270	1,740	5,060
8	1,265	1,225	3,475	985	384	3,710
	1,589	918	3,135	2,160	486	3,030
	791	466	1,180	2,085	276	411
11	204	172	384	270	64	295
	647	702	1,460	765	178	753
	262	591	1,750	395	100	805
14	235	355	1,035	296	168	669
	1,960	607	1,700	1,035	283	816
	445	614	3,315	554	92	1,022

The obtained data were compared with corresponding values from rats injected with either normal globulin or anti-liver globulin. There is a slight prolongation of the half-life for the pancreas as compared with the "normal" globulin series (2.40 days), but an identity with the anti-liver series (3.0 days). Otherwise there is a significant shortening of the half-life for the liver (2.8 days) as compared with the "normal" (3.71 days)

and anti-liver series (4.9 days). Under the conditions of this experiment it is difficult to reach a conclusion as to the specificity of radioactivity in the pancreas. Experiments were continued with various lots of anti-rat-pancreas globulin on several groups of rats with a total of 59 animals, but only in one group of rats were the results indicative of a significant accumulation of radioactivity in the pancreas. Comparative evaluation of radioactivity was made on the eighth day when the counts for the pancreas were 9,478 cpm/g. as compared with 1,600 cpm/g. for the liver and resulting in a pancreas to liver ratio (T/T) of 5.92. This selective localization in the pancreas is the more significant as the radioactive material was in this case injected intra-abdominally whereby the liver and the pancreas were equally exposed.

Physiological effect of the anti-pancreas serum. In the light of the introductory remarks on the physiological responses of the organ to the action of the homologous antiserum, rats were injected intra-abdominally with whole unaltered anti-rat-pancreas serum. A single dose of 1–2 ml. undiluted serum was injected in each rat and blood samples were tested for glucose by Folin-Malmros micro-method at the end of 18 hours fasting period. The range of blood glucose in seven untreated normal rats was 80–135 mg. per cent whereas 17 rats treated with anti-pancreas serum showed within 90 minutes a rise in blood glucose from 150 to 250 mg. per cent. No rise in glucose levels noted in rats treated with 2 ml. normal rabbit serum or with anti-liver rabbit serum. Further investigation showed that the hyperglycemia which reached its peak at 90 minutes is a temporary reaction which terminates with normal glucose values within the next 24 hours. No correlation between the precipitin titer and the activity of the antiserum *in vivo* was found.

Discussion. The anti-rat-liver serum produced by immunizing rabbits with whole liver homogenate contains different groups of antibodies, some of which are responsible for specific localization in the liver. These phenomena depend largely on the specificity and stability of the radioiodinated antibodies which are preferentially picked up by the homologous tissue. However, cross-reactions with other tissues indicated some kidney and lung localizing antibodies. Pressman, Sherman and Korngold (1951) were unable to trace liver localizing antibodies when whole rat

liver homogenate was used as antigen, but were successful in demonstrating localization when rat liver blood vessels were used for immunization. We have reason to believe that the vascular beds of the perfused rat liver were among the components of our whole liver homogenate against which localizing antibodies were formed.

It has been shown by Breinl and Haurowitz (1932) who first introduced iodination of antisera that excessive iodination decreased their serological specificity. With this in mind careful iodination methods were developed to safeguard the serological activity of radioiodinated antibody molecules. As mentioned before the material used by us is iodinated at a concentration of approximately 1 atom iodine per 1 molecule of globulin, whereas the introduction of 18 atoms of iodine per antibody molecule still retains its serological activity (Pressman and Sternberg, 1950). Nevertheless, the stability *in vitro* does not necessarily predict that the radio-bound compound will retain stable *in vivo*. To the contrary, the radioactivity carrier (antibody) injected intracardially rapidly reaches the tissues, and is involved in their metabolic activities at a rate characteristic and specific for the respective organ. The degree of this involvement will influence the biological decay and the half-life of the tagged protein with a possible splitting of iodine from the radioactive globulin. Thus we have to admit the possibility that theoretically not all registered radioactivity accounts for the radio-bound antibody, but the inorganic iodine once liberated is either taken up in the thyroid or excreted in the urine.

Since the rat is injected with heterologous proteins (rabbit globulin) which also act as antigen inducing the formation of antibodies, it is highly probable that the disappearance of the foreign protein is influenced in addition to the elimination rate of the specific organ also by the newly formed antibodies. This would probably coincide with the second week after injection when an abrupt fall in radioactivity in blood and tissues was not infrequently observed.

The relatively weak localization of radioactivity in the pancreas is perhaps correlated with the structural peculiarities of the rat pancreas, its mucoid consistency and, above all, with the very low vascularity. The scanty vascular bed as a weak antigen apparently does not elicit sufficiently active localizing antibodies

in the immunized rabbit. Nevertheless, the anti-pancreas serum induced a striking physiological effect in producing a high degree hyperglycemia by a single dose.

Summary

In immunizing rabbits with the whole rat liver homogenate localizing antibodies were formed and their presence revealed in the radioiodinated globulin fraction of the antiserum. The distribution of the anti-liver radioactivity carrier (antibody) in the injected rat showed a relative accumulation in the liver tissue although extensive cross-reactions with tissues of heterologous organs (lung, spleen, kidney) were recorded. Basic data on radioactivity concentration in tissues were obtained from counts per minute per gram of wet perfused tissue of the injected rat. The relative concentration of radioactivity was evaluated by a T/T ratio in dividing the counts for the homologous tissue over the counts of any heterologous tissue. In using the T/T ratio it is found that the accumulation of radioactivity in the liver of rats injected with anti-liver globulin exceeds that of other organs of the same rats. The half-life calculated over an observation period of 19 days from counts plotted logarithmically against time was 4.9 days for the liver and 2.4 days for blood. An anti-rat-pancreas serum (precipitin test titer up to 1:10,000) was obtained with the whole rat pancreas as antigen. Distribution of radioactivity in rats injected with the globulin fraction of the anti-pancreas serum revealed an accumulation in the pancreas when the radioactive material was injected intra-abdominally. Otherwise the radioactivity in the pancreas was not remarkable. Anti-rat-pancreas serum when injected into rats, induces an immediate hyperglycemia with a peak of 250 mg. per cent within 90 minutes. The reaction seems to be specific since antiliver serum or normal rabbit serum had no effect on the blood glucose levels of the injected rats.

Acknowledgment. Thanks are due to William Hardin, medical student, for his valuable assistance in the preparation of the manuscript.

REFERENCES

Anigstein, L., and C. M. Pomerat: Reticulo-endothelial Immune Serum (REIS). IV. Experimental Production of Anemia and Bartonellosis in Rats by Inhibitory Dosage. *Tex. Rep. Biol. and Med.*, 3: 545–557, 1945.

Anigstein, L.: Radioactive Isotopes as Tracers of Antibodies. *Tex. Jour. Sc.*, V: 148–153, 1953.

Besredka, A.: La leucotoxine et Son Action. *Ann. Inst. Pasteur*, 14: 390–401, 1900.

Bogomolets, A. A.: Antireticular-cytotoxic Serum as a Means of Pathogenic Therapy. *Am. Rev. Sov. Med.*, I: 101–112, 1943.

Breinl, F., and F. Haurowitz: Aenderungen der Specifität von Immunseren nach chemischer Vorbehandlung. *Zeitschr. f. Immun. Forsh.*, 77: 176–186, 1932.

Eisen, H. N., and D. Pressman: The Zone of Localization of Antibodies. VIII. *Jour. Immun.*, 64: 487–498, 1950.

Eisen, H., B. Sherman, and D. Pressman: The Zone of Localization of Antibodies. IX. The Properties of Anti-Rat-Lung Serum. *J. Immun.*, 65: 543–558, 1950.

Frieden, E. N., C. M. Pomerat, and L. Anigstein: Identification of the Inhibitory Factor of Reticulo-endothelial Immune Serum (REIS) in a Globulin Fraction. *Science*, 102–354, 1945.

Greene, E. C.: Anatomy of the Rat. *Trans. Am. Phil. Soc.*, 27: pp. 370, 1935.

Henle, W., L. A. Chambers, and V. Groupe: The Serological Specificity of Particulate Components Derived from Various Normal Mammalian Organs. *J. Exp. Med.*, 74: 495–510, 1941.

Lansteiner, K.: *The Specificity of Serological Reactions.* Harvard Univ. Press, pp. 310, 1945.

Latta, H.: Experimental Hypersensitivity in the Rabbit. Blood and Tissue Concentrations of Foreign Proteins Labeled with Radioactive Iodine and Injected Intravenously. *J. Immun.*, 66: 635–652, 1951.

Lippman, R. W., H. U. Marti, and D. H. Campbell: Nephrotoxic Globulin Nephritis. I. Course After a Single Intravenous Injection. *A.M.A. Archives of Pathology*, 53: 1–14, 1952.

Metchnikoff, E.: Sur les Cytotoxines. *Ann. Inst. Pasteur*, 14: 369–377, 1900.

Pomerat, C. M., E. H. Frieden, and Enid Yeager: Reticulo-endothelial Immune Serum (REIS). V. An Experimental Anemia in Bartonella Infected Rats Produced by Anti-blood Immune Serum. *Jour. Inf. Dis.*, 80: 154–163, 1947.

Pressman, D., and G. Keighley: The Zone of Activity of Antibodies as Determined by the Use of Radioactive Tracers; the Zone of Activity of Nephritoxic Anti-kidney Serum. *Jour. Immun.*, 59: 141–146, 1948.

Pressman, D.: The Zone of Localization of Antibodies. IV. The *In Vivo* Disposition of Anti-Mouse-Kidney Serum and Anti-Mouse-Plasma Serum as Determined by Radioactive Tracers. *J. Immun.*, 63: 375–388, 1949.

Pressman, D., and B. Sherman: The Zone of Localization of Antibodies. XII. Immunological Specificities and Cross Reactions in the Vascular Beds of Liver, Kidney, and Lung. *J. Immun.*, 67: 21–33, 1951.

Pressman, D., B. Sherman, and L. Korngold: The Zone of Localization of Antibodies. XIII. *J. Immun.*, 67: 493–500, 1951.

Witebsky, E.: Die Serologische Analyse von Zellen und Geweben. *Naturwiss.*, 17: 771, 1929.

REACTIONS OF CELLS IN TISSUE CULTURE TO *HEMOPHILUS PERTUSSIS**

I. Effect of Whole Organisms on Brain Tissue and Results of Treatment with Specific and Normal Serum

Harriet M. Felton, Aldo Gaggero,† and C. M. Pomerat

Reports of severe reactions and even death following routine immunizing doses of *H. pertussis* vaccine in young children (Byers and Moll, 1948) have caused some physicians to question the wisdom of using currently available antigens for active protection against whooping cough. Progress in therapy has not been as spectacular as the results of widespread active prophylaxis. It is imperative, therefore, to continue to search for safer immunizing agents.

The *Hemophilus pertussis* organism is a small cocco-bacillus with very exacting growth requirements and is readily killed by minute changes in its environment. It is possible to maintain *H. pertussis* in a virulent state by lyophilization and the organism has been fractionated by immunochemical means (Flosdorf and Kimball, 1940). The process begins by disrupting the bacteria by sonic vibration. The sonic extract contains the antigenic elements, the endotoxin as well as the surface agglutinogen. It has been possible to remove gradually the toxin from successive fractions by various extraction processes. The final product is a purified fraction, antigenically active in small doses, which contains no toxin (Flosdorf and McGuinness, 1942). Antiserum of high titer can be obtained after repeated doses of *H. pertussis* vaccine (McGuinness, *et al.*, 1940). This serum has been used for treatment of pertussis and in combination with the newer antibiotics is currently considered the therapy of choice. With the laboratory and clinical information concerning whooping

* From the Department of Pediatrics and the Tissue Culture Laboratory of The University of Texas, Medical Branch, Galveston. This study was supported by a grant from the Research Division of Sharp & Dohme, Division of Merck & Co., Inc. This report was presented before the Society for Pediatric Research at Buck Hill Falls, Pa., May 6, 1954.

† Rockefeller Foundation Fellow, Instituto Bacteriologica de Chile, Santiago, Chile.

cough now available, combined with a reliable system of tissue culture technique it may be possible to augment the existing knowledge of the histopathology of the disease. The mechanisms by which the organism produces its damage as well as further facts concerning the components of the organism responsible for the changes might also be determined.

Materials and Methods

Hemophilus pertussis. The Kendrick strain No. 18323 which has been widely used in standardization of vaccines was selected. The culture was regenerated from the lyophiled state and planted on Bordet-Gengou medium. Throughout these studies transfers were made after 48 hours to produce each culture generation and organisms from the third generation were used. Suspensions of live and heat-killed bacteria were prepared in Gey's balanced salt solution in a concentration of 2 billion organisms per ml. The suspension of live organisms was used within one hour because of the rapid death of the organisms in any environment other than the specialized medium. To heat kill the bacteria the suspension was placed in a 60° C. water bath for 1 hour. Both antigens were introduced into the roller tube nutrient in a final dilution of 1:10.

Specific antiserum. This was prepared by pooling samples of high agglutinin titer obtained from babies following routine pertussis immunization. The titer of the pool was 1:2560. The *normal serum* was obtained from adults who had no history of pertussis or immunization. They were skin test negative and showed no agglutinins by routine testing.

Brain tissue. This was obtained from sterile autopsies performed within 2 hours after a baby or fetus became available for study. Four different brains were used in this series of experiments: a three month fetus from a spontaneous abortion, a five month fetus delivered by Caesarian section from an eclamptic mother, a premature infant of seven months gestation who expired 12 hours after delivery, and a stillborn full term infant who expired just before delivery. It is advisable to obtain the tissue as soon after death as possible and to begin the preparation of the specimens for culture at once. In a few instances, however, the cultures have grown well when the fetus had to be refrigerated overnight and the explants cultured the next morning. If there is delay after the brain has been removed it can be kept in a sterile container, suspended or moistened with Gey's balanced salt solution.

Tissue culture methods were those currently employed in this laboratory (Pomerat, *Methods in Medical Research,* 1951). The handling and interpreting of outgrowth from explants of nervous tissue was largely based upon experiences as described by Costero and Pomerat (1951) and Hild (1954).

The tissue was prepared for culture with aseptic precautions. Small fragments 2 x 2 mm. were cut with sharp, slender knives and placed in fresh Gey's solution to be washed. Three to five pieces of tissue were placed, each in a drop of plasma, on a narrow coverslip (No. 1 thickness, 12 x 50 mm.).

Embryonic extract was added to form a plasma clot completely surrounding all the explants. Two coverslips were placed back to back and gently slipped into an empty sterile roller tube (16 x 150 mm.) and closed with a sterile cotton plug. After the clot had set for about 2 hours, 2 ml. of a sterile nutrient medium containing 50 per cent human ascitic fluid, 45 per cent Gey's balanced salt solution and 5 per cent chick embryonic extract was added. This solution also contained Penicillin G sodium in a concentration of 1:1000. The roller tubes were sealed with sterile rubber stoppers and placed in a revolving drum (8 revolutions per hour) maintained at a temperature of 37° C. The nutrient medium was completely replaced twice a week. The tissue cultures were inoculated with the bacteria when sufficient outgrowth was seen (4–12 days).

The effect of the different antigens used in these experiments was studied by: (1) fixing roller tube preparations at stated intervals, staining with Jacobson's method (May-Grunwald, Giemsa mixture); (2) phase contrast photomicrography in closed chamber preparations; and (3) continuous time lapse cinematography in perfusion chamber preparations (Pomerat, *Methods in Medical Research,* 1951). Still pictures were taken with 8 mm. dark M. and 1.8 mm. dark M. oil immersion phase objectives. The closed chamber was prepared by sealing the coverslip holding the tissue bathed in nutrient medium to a glass slide with paraffin. Periodic illumination of the perfusion chamber was synchronized with the exposure mechanism of the moving picture camera so that a continuous record was made at either 8 or 1 frames per minute, depending upon the stage of the experiment. The same 8 mm. dark M. and 1.8 mm. dark M. oil immersion phase objectives were used. A two hour base line at low power was recorded before the organisms were added.

Procedure

Human brain tissue. Premature or newborn cerebellum and cortex were inoculated with suspensions of live and heat-killed *H. pertussis* 0.2 ml. in 1.8 ml. of nutrient medium. The control tubes were inoculated with Gey's solution alone in the same proportion. After 24 hours tubes containing each antigen, as well as control tubes, were fixed and stained. At this time hyperimmune and normal serum (0.2 ml.) were added to the remaining tubes. The action of the sera was observed by fixing and staining 24 and 48 hours later, At the end of the experiment the tissue culture had been exposed to the bacteria for a total of 72 hours.

Cat brain tissue. Newborn kitten cerebellum was prepared and set up using the same methods as for human brain tissue. The same inoculation schedule was followed.

Results

Human brain tissue: in roller tubes.

Bacteria alone. In comparison with 170 control explants (Plate II, Figs. 3, 4), there was unmistakable evidence of damage to cells seen in 280 explants from the infected preparations.

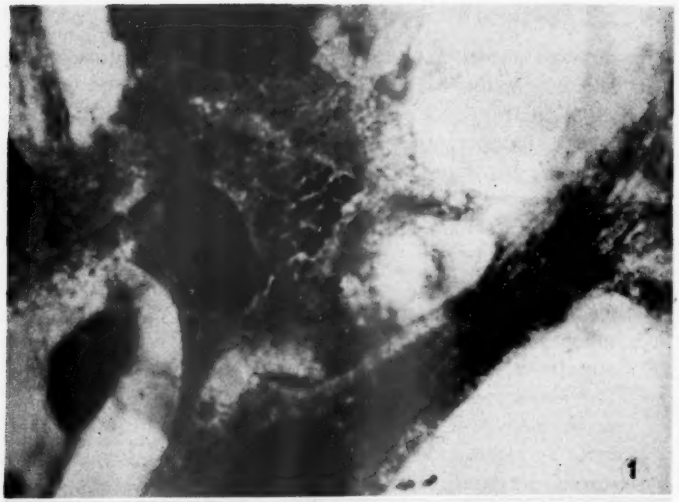
This damage was more marked following the inoculation of live bacteria than with heat-killed organisms. Cell damage was indicated by (a) presence of bacteria within the cytoplasm (Plate I, Fig. 1), (b) increased vacuolization (Plate I, Fig. 2), frequently with bacteria within the vacuoles, (c) nuclear deformation in proportion to the severity of the damage (Plate II, Fig. 1, 2), (d) decrease in the number of cells showing mitosis. These changes were not seen in every cell in the culture, but the number of cells showing these changes as well as the extent of the damage increased directly with the length of time the tissue was in contact with the antigen alone. The types of cells involved in this pattern were probably of mesenchymal origin. Granule cells and astrocytes can often be seen showing these changes.

Specific antiserum. The addition of antiserum to 160 explants following inoculation with *H. pertussis* seemed to check the progress of the damage (Plate III, Figs. 1, 2). In 24 hours there was improvement in the appearance of the cells and after 48 hours the cells showed very little damage. The preparations containing the bacteria, but no antiserum, were severely damaged at the end of the same observation period.

Normal serum (control). In 160 explants, the damage seen after inoculation with *H. pertussis* did not appear to be decreased by the addition of normal serum (Plate III, Figs. 3, 4). Instead, the process continued on a downward course, so that 48 hours after normal serum had been added the damage was as great as at 72 hours after bacteria alone.

The specificity of the apparent effect of *H. pertussis* in human and cat brain tissue was checked in a limited series of observations in other types of tissue, such as 12 day-old chick embryo heart muscle, leg muscle, and skin, although minimal changes were observed in the 18 day-old chick embryo brain. A heavy challenging dose of organisms was introduced into the Hela cell strain (a cervical carcinoma) without the slightest evidence of damage even after 96 hours. Plans for the study of *H. pertussis* in respiratory tissue have been made and preliminary experiments begun.

Attempts to recover *H. pertussis* from the roller tube preparations at the various times at which the tissues were fixed, were for the most part unsuccessful. Occasionally after long centrifugation of the nutrient medium a few colonies could be grown



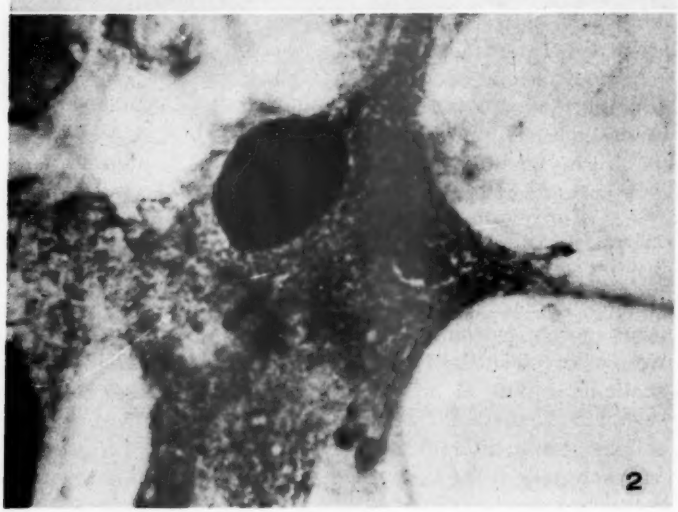


PLATE I

FIG. 1. Cell showing the organism in the cytoplasm. Five day-old human cerebellum culture. Twenty-four hours after infection with live H. pertussis. Jacobson's stain. 1630x.

FIG. 2. Organism within a vacuole in the cytoplasm. Five day-old human cerebellum culture. Twenty-four hours after H. pertussis infection. Jacobson's stain. 1630x.

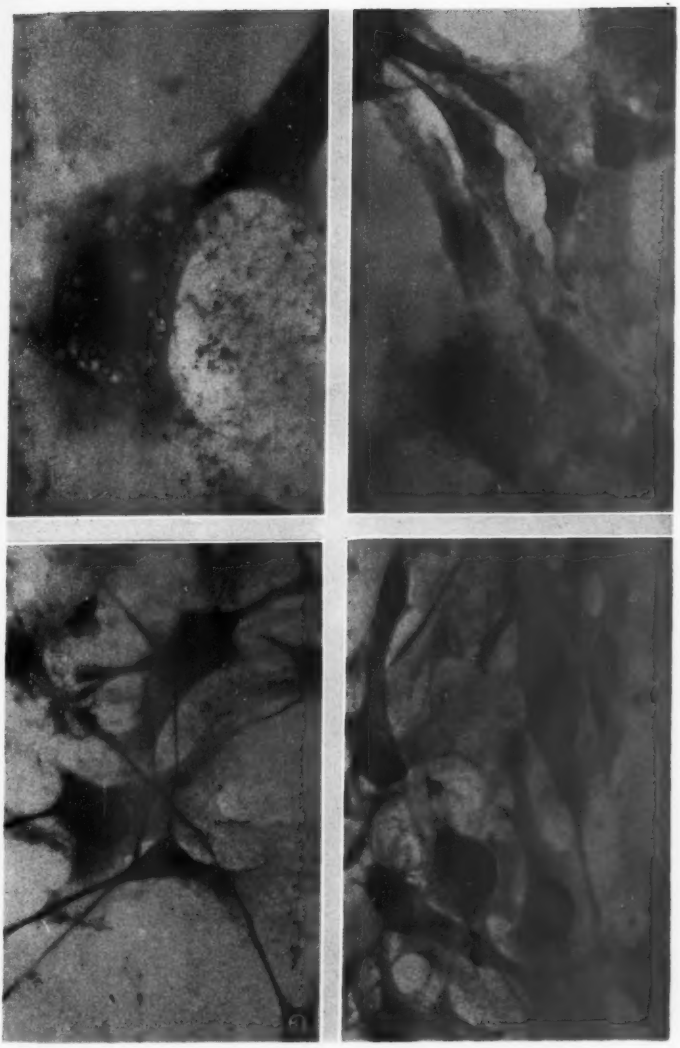


PLATE II

FIGS. 1 and 2. Six day-old human cerebellum culture, showing cells with nuclear deformation. Forty-eight hours after infection. Jacobson's stain. 520x.

FIGS. 3 and 4. Six day-old human cerebellum culture, 48 hours as a control for figures 1 and 2. Jacobson's stain. 520x.

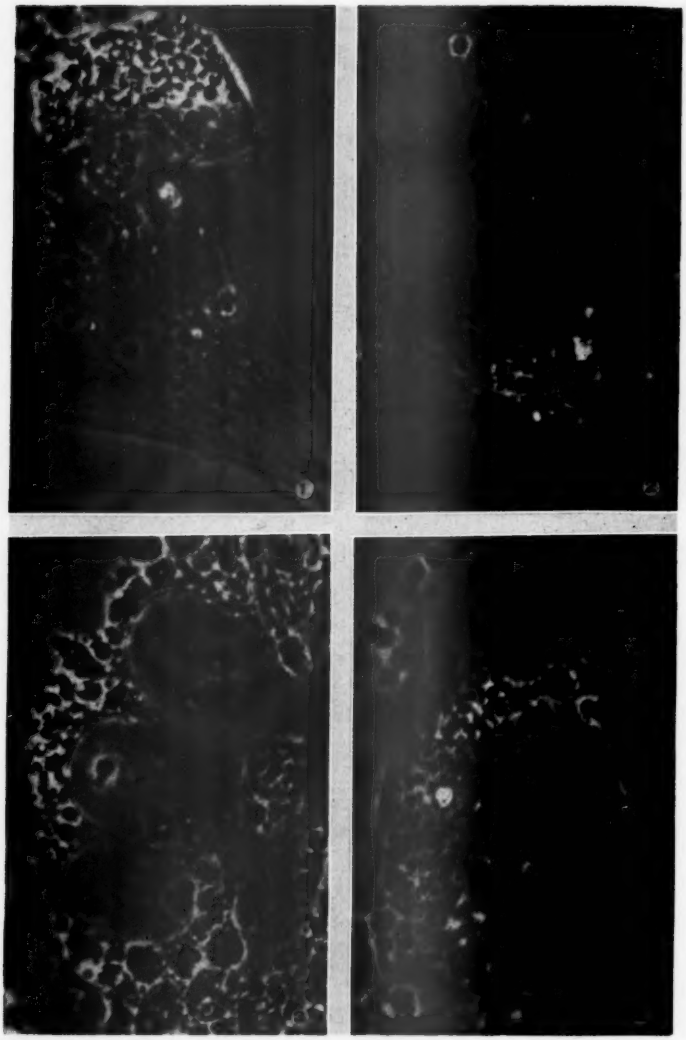


PLATE III

FIGS. 1 and 2. Eleven day-old human cerebellum culture infected for 24 hours and then treated with hyperimmune serum for 24 hours. Cell membrane activity, mitochondria, juxta-nuclear zone. Phase contrast photomicrography. 1250x.

FIGS. 3 and 4. Eleven day-old infected human cerebellum culture infected for 24 hours and then treated for 24 hours with normal serum, no cell activity, in figure 4 bubbling in the cell membrane. Phase contrast photomicrography. 1250x.

on Bordet-Gengou medium. The bacterial suspensions used were very light, the number of organisms having been arbitrarily chosen after considering the infecting dose and total blood volume of a 10 gram mouse. It appeared that most of the organisms were engulfed by the tissue cells within 24 hours. Very few organisms were seen elsewhere on the slide and the nutrient medium was clear even after 7 days in some instances without the supernatant having been changed. In order to produce bacterial multiplication as well as to maintain the tissue culture a modified Cohn-Wheeler liquid pertussis medium was tried in place of the regular nutrient. It was possible to recover the bacteria periodically, but the tissue in both control and inoculated preparations showed poor outgrowth and loss of definite cell outline after five days.

Human brain tissue in Perfusion Chamber. Three periods of continuous observation for 48 hours using the perfusion chamber showed that the activity and normal functions of the cell, seen before the organisms were added, progressively decreased until at 24 hours the entire preparation was practically motionless and the cell outlines looked shrunken. There was also a granular appearance of the entire preparation not seen in the control. Almost immediately after the addition of the specific antiserum the motility of the whole field began to be reactivated. Under high power oil immersion the individual cells showed resumption of nuclear constriction and increased activity in the juxtanuclear zone (zone of Golgi). There was reappearance of cell margin undulation. These changes did not appear when normal serum was added.

Cat brain tissue in Roller Tubes. Experiments with cat cerebellum were not as extensive as with the human brain tissue. The purpose of the trials with live and heat-killed bacteria was only to find a more easily available test tissue. The changes seen in the examination of 400 explants were similar to those seen in the human brain preparations (Plate IV, Figs. 1, 2). There was less vacuolization in the cytoplasm of the inoculated preparation than in the human tissues and the nuclear deformation resulted in a crescent-like structure (Plate V, Figs. 1, 2) rather than showing notching and constriction on both sides characteristically seen in the human tissue (Plate II, Figs. 1, 2).

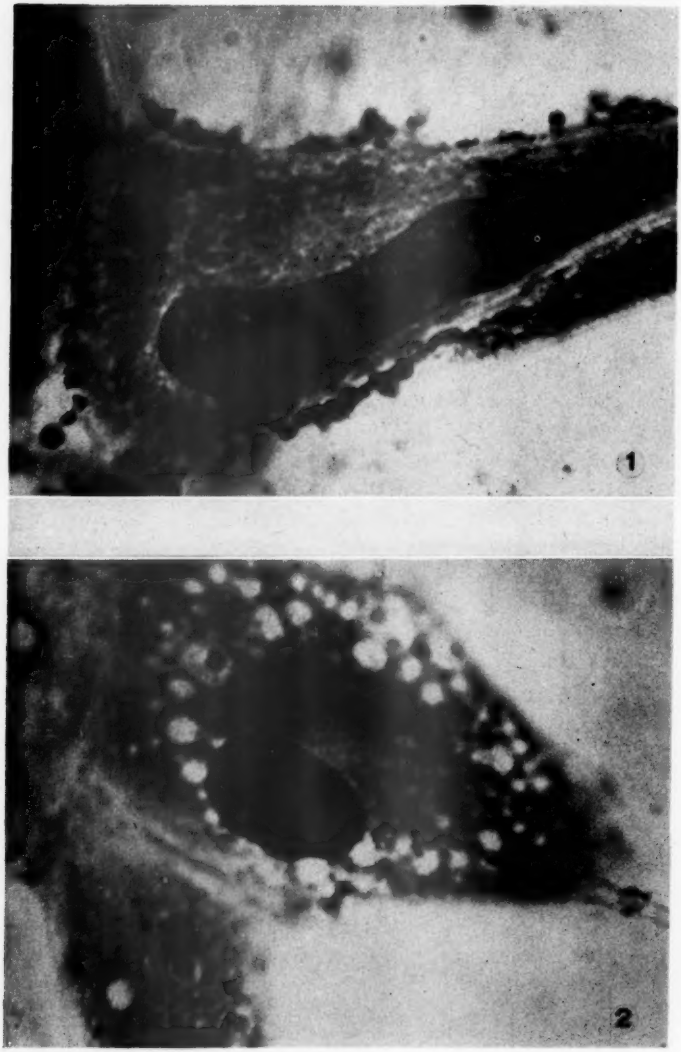


PLATE IV

FIG. 1. Twelve day-old kitten cerebellum culture 48 hours after infection with H. pertussis. Bacteria on the cell surface. Jacobson's stain. 1630x.

FIG. 2. Twelve day-old kitten cerebellum culture 48 hours after infection with H. pertussis. Cell showing vacuoles and a few bacteria within the cytoplasm. Jacobson's stain. 1630x.

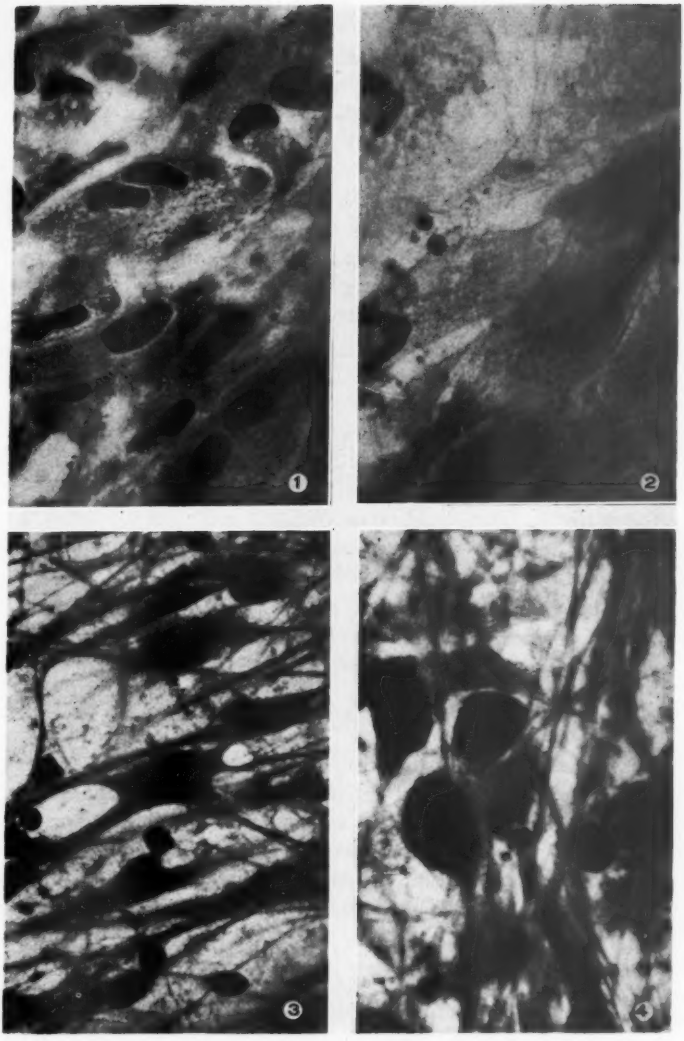


PLATE V

FIGS. 1 and 2. Twelve day-old kitten cerebellum culture infected for 72 hours with H. pertussis, cell showing nuclear deformation; in figure 2, a cell showing bacteria. Jacobson's stain. 520x.

FIGS. 3 and 4. Twelve day-old kitten cerebellum culture, control showing cells with normal appearing nuclei. Jacobson's stain. 520x.

Discussion. Advances in tissue culture techniques have presented fresh opportunities for the study of the mechanisms of bacterial infections at the cellular level. With the use of phase contrast photomicrography and continuous time lapse cinematography in combination with perfusion techniques, the findings may be documented and recorded in such a way that the results can be examined at any time during a continuous experiment. In these experiments by challenging a cellular outgrowth of tissue with measured doses of bacteria, the pattern of cell damage observed in the preliminary experiments has been reproduced in each subsequent trial.

The nutrient solution used throughout the work was prepared with human ascitic fluid. The source of this material varied from time to time. There was no attempt to determine its steroid content. In spite of the unknown factors present in the human malignant transudates used to maintain the cells in culture the findings could be reproduced and even predicted. In the future it is hoped that the use of a synthetic nutrient medium will eliminate this controversial variable.

The mechanism by which the bacteria enter the cells is not yet clear, and the details of the effect of the antiserum in halting the damage to the tissue cells must be investigated very carefully.

Conclusions

H. pertussis produces unmistakable and reproducable damage in cultures of human and cat brain tissue. Such changes have not been produced in the chick embryo heart muscle, leg muscle or skin nor in Hela cells under the similar experimental conditions. The introduction of specific antiserum appears to halt progress of the damage produced by the bacteria. Normal serum does not produce this effect.

Acknowledgments. Grateful acknowledgment is made to Mr. George Lefeber and Mr. Earl Pitsinger for their help in preparing the photomicrographic records, to Miss C. Y. Willard for preparing and maintaining the pertussis cultures, and to Dr. F. B. Engley for his counsel and encouragement.

REFERENCES

Byers, R. K., and F. C. Moll: Encephalitic Symptoms Following Pertussis Immunization. *Pediat.*, 1: 437–458, 1948.

Costero, I., and C. M. Pomerat: Cultivation of Neurons from the Adult Human Cerebral and Cerebellar Cortex. *Am. J. Anat.*, 89: 405–467, 1951.

Flosdorf, E. W., and A. C. Kimball: Separation of Phase I agglutinogen of *H. pertussis* from Toxic Components. *J. Immunol.*, 39: 475–494, 1940, No. 6.

Flosdorf, E. J., and A. C. McGuinness: The Antigenic Structure of Haemophilus Pertussis and Its Clinical Significance. *Am. J. Dis. Child.*, 64: 43–50, 1942.

Hild, W., Das morphologische kinetische und endokrinologische verhalten von hypothalamischem und neurohypophysärem gewebe *in vitro. Zeits. für Zellforschung*, 40: 257–312, 1954.

McQuinness, A. C., W. C. Bradford, and J. G. Armstrong: The Production and Use of Human Hyperimmune Whooping Cough Serum. *J. Pediat.*, 16: 21–27, 1940.

Pomerat, C. M.: Tissue Culture Methods. *Methods in Medical Research*, Year Book Publishers, Chicago, 1951, Vol. 4: 198–291.

PSYCHOTHERAPEUTIC ADEQUACY AND JUDGEMENT OF PERSONALITY

H. A. GOOLISHIAN*

In a summary of several research reports (Fiedler, 1953), it has been suggested that there is a difference between psychodiagnostic and psychotherapeutic understanding. These reports also suggest that "good" psychotherapists seem to have much in common with the subjects in Tafts' (Taft, 1950) study who made inaccurate or poor social judgements. In one sense these are remarkable and interesting findings. It could be hypothesized that a good psychotherapist is an expert in interpersonal and social relations and as such would make accurate and careful predictions or diagnoses of social structure or personality characteristics. Fiedler (Fiedler, 1953), on the other hand, offers the hypothesis that psychotherapeutic understanding may be a feeling reaction on the part of the therapist and that this feeling reaction, so important to adequate psychotherapeutic relationships, may be entirely unrelated to the ability to judge others accurately or predict their behavior. The present study was designed to explore this hypothesis.

Method

The subjects for this experiment consisted of four psychiatric residents at the Department of Psychiatry, The University of Texas, Medical Branch. They were selected from a larger group of eleven residents on the basis of supervisors ratings of therapeutic adequacy. All subjects had completed at least two years of psychiatric training, had known and worked with each other, and were actively engaged in supervised psychotherapy. In conjunction with another research project the subjects were asked to rate all eleven members of the resident group on the basis of Fromm's character types (Fromm, 1947), (Productive, exploitive, hoarding, marketing, and receptive characters). These ratings were accomplished by means of paired comparisons and each rater was supplied with a summary of the five character types as described by Fromm.

Two control therapists were asked to independently rank the group of eleven training psychotherapists on the basis of their subjective estimations

* From the Clinical Psychology Service of the John Sealy Hospital, The University of Texas Medical Branch, Galveston. Received for publication June 20, 1954.

of overall psychotherapeutic adequacy.[1] These supervisor ranks were pooled and the two therapists ranked as most adequate along with the two therapists ranked as least adequate were selected for use in this study. The experimental subjects were then intercorrelated with each other on the basis of their rankings of the personality characteristics of the larger group of eleven. These rankings included an estimation of the residents own position within the group.

While this procedure may not be diagnosis in a strict clinical sense it was felt that it involved many common elements. In effect each of the subjects was faced with the task of "diagnosing" each member of the group in terms of Fromms' character types. This is similar to the diagnostic task of the clinician who must evaluate the personality characteristics of an individual patient and who must of necessity use as his reference scale diagnostic stereotypes against which he evaluates his patients.

Findings

The correlations presented in table 1 were computed by means of the Pearson product-moment formula and represent the intercorrelations of the four therapists comprising the experimental populations. It should be kept in mind that these correlations are

TABLE 1

Therapists Intercorrelations

Therapist	1	2	3	4
1		.19	.01	.00
2	.19		.36	.24
3	.01	.36		.24
4	.00	.24	.24	

not the usual "r" technique wherein two tests or measurements are correlated on a population of people but are "Q" correlations: the correlation of people on a population of measurements. Thus the correlations in table 1 are a measure of similarity between the experimental subjects, that is, a measure of their agreement in rating the personality characteristics of the group. Therapists one and two are the most adequate therapists and therapists three and four the least adequate according to pooled supervisors' ratings. Both supervisors agreed on the rank positions of the four therapists in the total group of eleven.

[1] The author thanks Drs. E. McDanald and W. Shanahan for their cooperation in this research.

Discussion. If it were assumed that adequacy as a psychotherapist was related to the ability to accurately diagnose the personality of others it would follow that the most adequate psychotherapists would show marked agreement with each other on independent estimations of personality characteristics. From this it would also follow that less adequate therapists would demonstrate little agreement or correlation with more superior therapists. Fiedler, however, suggests that therapeutic adequacy and diagnostic adequacy are somewhat unrelated. The present study supports this hypothesis. The most adequate therapists show no more agreement with each other than do inadequate therapist. Further, the correlations between the inadequate and adequate therapists are within the same range as are the other correlations. Indeed, the correlation of .36 between the least adequate and the most adequate therapist is the highest correlation found.

These findings must, of course, be held as tentative. An absolute scale of the personality characteristics of the group is lacking and the study in no way measures the absolute diagnostic accuracy of the therapists. The findings do suggest, however, that ability to assess personality of a peer group does not adequately differentiate between adequate and inadequate therapists. This finding is consistent with the hypothesis of Fiedler and is taken as further evidence for the position that diagnostic skill and therapeutic skill are probably different attributes.

Summary. Four psychotherapists, two ranked as adequate and two ranked as inadequate by supervisors, were intercorrelated on the basis of their personality ratings of peers. Adequate therapists correlated no better with each other than they did with less adequate therapists. This was taken as supportive evidence for the hypothesis that psychodiagnostic skills and psychotherapeutic skills represent different spheres.

REFERENCES

Fiedler, F. E.: Quantitative Studies of the Role of the Therapists Feelings Toward Their Patients. In O. H. Mower (ed.) *Psychotherapy: Theory and Research.* Ronald Press, New York, 1954.

Fromm, Erich: *Man for Himself.* Rinehart, New York, 1947.

Taft, R., Some Correlations of the Ability to Make Accurate Social Judgments, Unpublished Ph.D. Dissertation. University of California (Berkeley), 1950.

RESPONSE OF TRICHOMONAS HOMINIS TO CARBARSONE: A CASE REPORT FROM KOREA*

THOMAS A. HAEDICKE AND BOBBY JONES

Trichomonas hominis is a cosmopolitan flagellate that is supposed to be non-pathogenic for man. It is usually considered to be a harmless commensal which finds suitable culture material, during intestinal disturbances from any cause, to multiply profusely (Strong, 1944). It is difficult to state whether these organisms are pathogenic or not since they are found in the stools of people in perfect health and in those with diarrhea of unknown origin.

This flagellate lives and reproduces in the ileum and large intestine and any alterations in tissues should be found there (Shattuck, 1951). Flagellates have been found invading the glandular cells and connective tissue from sections of the intestine examined in a fatal case infected with Trichomonas hominis (Wenyon, 1926). Although the parasites are found in liquid stools it seems logical, therefore, to examine the intestine with the aid of a sigmoidoscope to follow the course of this infection.

The treatment of Trichomonas hominis is unsatisfactory and this infection may continue for many years (Shattuck, 1951). Clinicians have recommended such agents as carbarsone, emetine, acetarsone, treparsol, and chiniofon with little or no success in eradication of this flagellate. Soper (1943) reported the successful treatment of a few cases with stovarsol. It is the purpose of this paper to report a case from Korea that responded well to carbarsone therapy.

Case Report

A 23 year old white male soldier was admitted to the 11th Evacuation Hospital in Korea on 13 February 1952 with a history of intermittent diarrhea for five months. The onset of the diarrhea was abrupt with a bout of watery diarrhea lasting for about two weeks.

* From the Medical Service, Eleventh Evacuation Hospital, APO 301.
Received for publication June 15,1954.

During the initial two-week period, the patient had 8 to 12 loose watery stools daily, preceded by diffuse lower abdominal cramping pains. This initial period was treated with diarrheal mixtures only. For the past five months he suffered recurring bouts of diarrhea at frequent intervals. Tenesmus, flatulence, and explosive type diarrhea had been present just prior to admission. On several occasions the patient had the strong urge to defecate so quickly that he was incontinent. The last episode began approximately one and a half weeks prior to admission to the hospital. Except for the diarrhea and its accompanying related symptoms of weakness, tenesmus, and lower abdominal cramps, no other symptoms were present. The past medical history was non-contributory to the present illness. On admission his temperature was 99.4° F., pulse 80, and respiration 20. He was a well developed and well nourished patient, showing no signs of dehydration. The blood pressure was 122/82. Examination of the abdomen revealed no localized tenderness, palpable masses or rigidity. Rectal examination was negative.

On 14 February 1952 a sigmoidoscopic examination revealed the anal and sigmoid colon to have a fine, red, granular appearance which bled easily. No ulcers were observed. Tremendous numbers of trophozoites of Trichomonas hominis with red cells in them were observed. A biopsy of the mucosa revealed atrophic changes. The submucosa contained increased fibrous connective tissue and large numbers of lymphocytes and plasma cells. A few lobulated nuclei could be identified but it could not be determined whether these represented eosinophils or polymorphonuclear leukocytes. A stool examination revealed many trophozoites of T. hominis. The white count was 6,050 and the differential showed neutrophiles 63, monocytes 1, lymphocytes 33, and eosinophiles 3. The hemoglobin was 17.7 grams. Examination of urine was normal.

Beginning on 17 February 1952 aureomycin 2 gms. and chloroquine 0.5 gms. were given daily for ten days. Daily stool examination revealed varying numbers of the trophozoites of Trichomonas hominis present in each stool examination. The number of stools continued to average about ten each day. Lower abdominal cramps remained a prominent complaint. Since no improvement resulted from hexylresorcinol alone or from aureomycin and chloroquine in combination, carbarsone 0.25 grams two times daily for ten days was begun on 28 February 1952. Within twenty-four hours after institution of this therapy there was a decrease in the number of trophozoites seen on examination of the stools. Repeated stool cultures and smears failed to reveal any other intestinal parasites, viral or bacterial organisms. On 1 March 1952, the patient stated that for the first time in over five months his bowel movements felt normal. From the first of March until his discharge to duty on 13 March 1952 he had only one or two firm bowel movements daily without any tenesmus. Daily stool examinations after 1 March 1952 failed to reveal any trophozoites. After ten days of carbarsone therapy, the patient had no diarrhea or abdominal cramps. Final sigmoidoscope examination on 10 March 1952 revealed normal appearing mucosa, and smears were negative for trophozoites. He was discharged to duty on 13 March 1952.

Discussion. This case presents a characteristic clinical picture of a patient having diarrhea with Trichomonas hominis being present in his liquid stools. A number of sigmoidoscope examinations were performed during the course of his management in order to follow the changes in the intestinal mucosa.

It is felt that the diarrhea in this case was caused by the presence of Trichomonas hominis organisms within the intestinal mucosa. The diarrhea was present as long as the trophozoites of T. hominis were found. The sigmoidoscope examination before treatment with carbarsone showed that the intestinal mucosa had a fine, red, granular appearance which bled easily. A biopsy of the intestinal mucosa revealed atrophic changes and the submucosa revealed large numbers of lymphocytes, plasma cells and fibrous connective tissue.

The diarrhea and sigmoidoscope findings did not respond to treatment with hexylresorcinol or aureomycin and chloroquine. There was also no change in the number of active trophozoites in the stool examinations.

This patient showed an excellent therapeutic response to carbarsone therapy. His diarrhea and other intestinal symptoms disappeared rapidly and on completion of therapy he was having one to two well formed stools per day. Repeated stool examinations for trophozoites of Trichomonas hominis were negative 48 hours after carbarsone was begun. A sigmoidoscopic examination performed after carbarsone treatment was completed revealed normal appearing mucosa.

Summary

A case of Trichomonas hominis from Korea is reported. It is felt that the Trichomonas hominis organisms produced the changes in the intestinal mucosa and diarrhea in this case. Hexylresorcinol as well as aureomycin and chloroquine were found to be ineffective in the therapy of this case. A therapeutic response was attained with carbarsone. It is recognized, however, that this response could have been purely coincidental.

REFERENCES

Strong, R. P.: *Stitt's Diagnosis, Prevention and Treatment of Tropical Diseases.* The Blakiston Company, Philadelphia, Page 463, 1944.

Shattuck, G. C.: *Section on Intestinal Protozoa of Man, Diseases of The Tropics.* Appleton-Century-Crofts, Inc., New York, 1951.

Wenyon, C. M.: *Protozoology*. William Wood & Co., New York, 1926.

Soper, H. W.: Pathogenicity of Intestinal Protozoa. *AM. J. Digest. Dis.*, 10: 366, 1943.

TUMORS OF THE SALIVARY GLANDS

JOSEPH K. JOHNSON AND JOHN H. CHILDERS*

In clinical medicine tumors of the salivary glands are regarded as uncommon, usually benign lesions, and most often occurring in the parotid. These views are correct, such tumors constituting only about one per cent of all human neoplasms and being benign in about 75 per cent of cases. Yet this group of tumors is one of the most extensively studied and perennially interesting in the realm of pathology. Few pathologists, from the time of Virchow to the present day, have failed to be preoccupied to some extent by the histological diversification of these tumors. It is not surprising, therefore, that the student or the general practitioner, encountering one of these lesions and referring to the voluminous literature for further enlightenment, may well become confused by the bewildering names and seemingly endless varieties of these tumors.

Classification

Tumors of the major salivary glands have for some time been a subject of special interest to the Department of Plastic and Maxillofacial Surgery of this institution. As a result of their aggressive approach to these tumors a considerable number of surgical specimens have been submitted to the Department of Pathology. A review of one hundred and eighty of these tumors has been recently completed by the Laboratory of Surgical Pathology. This study included a review of the gross pathology, the original microscopic sections, freshly stained sections cut from the original paraffin blocks, and in some instances additional blocks of tissue taken from the formalin-preserved gross specimens. In some of the tumors not readily identifiable in the hemotoxylin and eosin sections special stains were employed. The special staining procedures found most helpful were the Masson's trichrome stain and Schiff's periodic acid stain.

This investigation was not intended to bring to light any hitherto undescribed variants of the numerous types of these tumors already known to exist. It did, however, provide several additional examples of some of the rare adenomas which have not been reported up to the present time. Two examples of the unusual, malignant lymphe-epithelioma described by Buxton (1953) are also reported. The principal achievement of this study was the

* From the Departments of Surgery and Pathology, The University of Texas, Medical Branch, Galveston. Received for publication August 2, 1954.

correlation of the various histologically distinct types with pertinent clinical data from the hospital records and from follow-up studies conducted by the University Tumor Clinic. This data has been tabulated and is presented below.

One of the secondary objectives of this study was to provide both the surgeon and the pathologist with a simple yet accurate classification designed to convey a maximum of histopathologic data without the use of complex and often ambiguous descriptive terminology. Since the most important question the surgical pathologist is called upon to answer is the question of malignancy in a given lesion he should render his diagnosis in terms universally recognized as connoting benignancy or malignancy. It is usually possible for the pathologist to determine whether malignant changes exist in a given lesion, therefore there seems little justification for the employment of terms suggesting a condition of intermediate or semi-malignancy. As it is now generally recognized that the great majority of neoplasms primary in the salivary glands arise from the epithelial components it would seem most logical to classify these tumors primarily as either adenomas or carcinomas. Simple, universally acceptable descriptive prefixes and appendages may be used to convey a very accurate picture of the histopathology. The fact that scientifically inaccurate terms such as "mixed tumor" and eponyms ("Warthin's tumor") are still current is doubly regrettable in view of the remarkable lack of unanimity in the minds of physicians concerning the biologic properties and potentialities of these lesions. It is the pathologist, however, who should take the lead in routinely using a more exact terminology.

TABLE 1

Epithelial Tumors Arising in the Salivary Glands

A. Adenomas
1. Simple Adenoma
2. Complex Adenoma
3. Adenoma with Lymphoid Stroma
B. Carcinomas
1. Muco-epidermoid Carcinoma
2. Adenocarcinoma
3. Epidermoid Carcinoma
4. Undifferentiated Carcinoma

Table 1 shows the basic classification used for the tumors encountered in this series. A discussion of the various histologically distinct tumors is given under the two major categories shown here. Table 2 shows the incidence of tumors of each type found in this series and in the larger series reported by Foote and Frazell (1953) from the Memorial Center for Cancer and Allied Diseases in New York.

Adenomas

In this series there were 127 adenomas of all types, an incidence of 70.6 per cent. This is in fairly close agreement with the findings in several other series recently reported (see Table 2).

Simple Adenomas are usually considered to be relatively rare neoplasms in salivary tissue and only two were encountered in our series, both being confined to the parotid salivary gland. One of these was of the oxyphilic cell

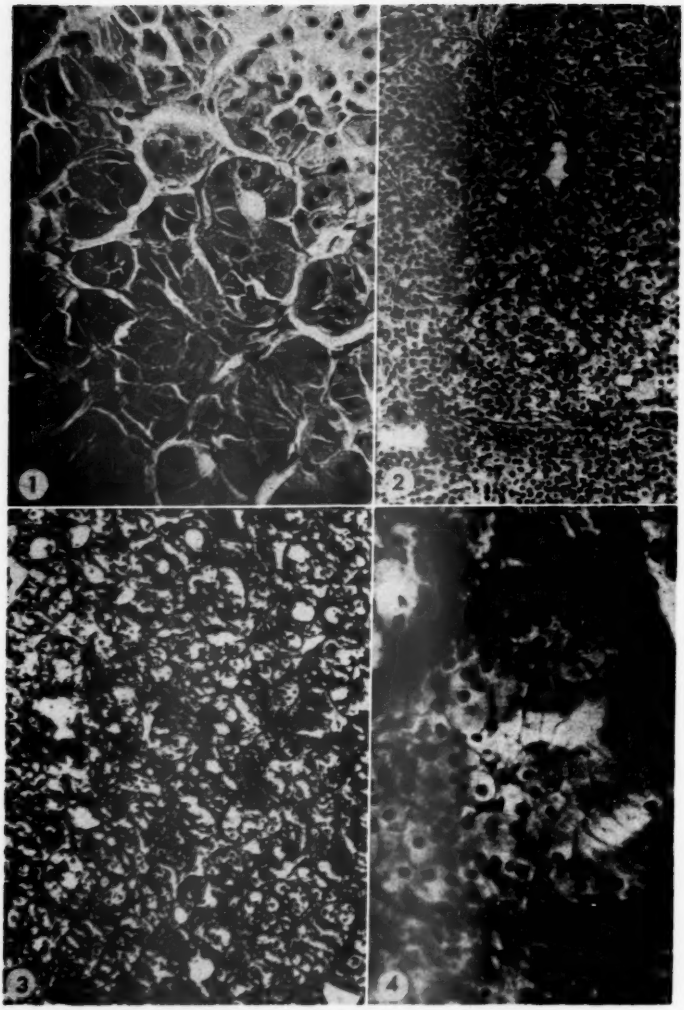
TABLE 2

Tumor Classification	No. of cases in present series		No. of cases reported by Foote and Frazell	
	Parotid	Submaxillary	Parotid	Submaxillary
Complex Adenomas (benign "mixed tumors")	96	23	447	47
Simple Adenomas				
Oxyphilic Cell	1	..	1	..
Acinic Cell	1	..	..	..
Papillary Cystadenoma Lymphomatosum	6	..	50	..
Sebaceous Cell	..	..	1	..
Mucoepidermoid Carcinoma	12	3	90	8
Adenocarcinoma				
Cylindroma Type	1	2	16	17
Acinic Cell Type	1	..	21	..
Papillary Type	1	..	..	..
Unclassified Including Adenocarcinoma arising in "mixed tumor"*	17	..	78	16
Epidermoid Carcinoma	8		26	13
Undifferentiated Carcinoma	4		30	6

* One case, not included in this table, was located in the palate.

type which arises from the ductal epithelium (Fig. 1). Such adenomas correspond to the oncocytoma of Hamperl and the oxyphilic or acidophilic granular cell adenomas described by Ackerman (1943) and Meza-Chavez (1949). Buxton (1953) has described a unique type which he called a "serous cell" adenomas and which he considered to be of acinic cell origin. One of the adenomas in this series was thought to be histologically similar to this (Figs. 2 and 3). The simple adenomas observed in this group on the average were rather small, circumscribed, soft, cellular tumors. They were completely excised and were not known to recur. Buxton thought he could identify certain of the adenocarcinomas in his series as being morphologically similar to ductal adenomas and acinic cell adenomas. We could draw no conclusions as to occurrence of malignant change in these adenomas, although there seems to be no theoretical objection to such a possibility. Despite the fact that these tumors tend to remain rather small and produce no symptoms it is generally agreed that they are best excised when first discovered. As they lend themselves readily to extirpation with the capsule intact recurrences are rare.

Complex Adenomas are the "mixed tumors" of common parlance. The term "complex adenoma" was suggested by Foote (1953) as a logical alternative



All photomicrographs were obtained from slides stained with hematoxylin and eosin, unless otherwise stated.

FIG. 1. Oxyphilic adenoma showing cords of large cells with granular cytoplasm. Attempted acinar formation is noted near the center of the field. X 270.

FIG. 2. Acinic cell adenoma with compactly arranged clusters of cells containing vacuoles in the cytoplasm. X 170.

FIG. 3. Acinic cell adenoma stained with periodic acid. Positive staining portions of the cytoplasm appear black in this photograph.

FIG. 4. Adenoma showing a cluster of cells which are arranged in a sebaceous gland-like manner.

to the older term, and it is used in preference to "Pleomorphic adenoma" which Willis (1948) proposed, though on strictly semantic grounds the latter term might be more exact. This is the most common type of salivary tumor encountered in any location, and is subject to so many bizarre morphological patterns that it is virtually impossible to catalogue all the possible variants. A single large tumor of this type may contain half-a-dozen distinctive patterns in different areas and there is so little practical correlation between cellular pattern and the incidence of malignant change that most authors do not attempt a more detailed classification. The extreme variability of structure is usually explained by the fact that under certain conditions of obstruction and irritation the proliferating ductal epithelium secretes large amounts of mucin thus produced will in time become inspissated, contract and deform the epithelium arranged in layers, or it may form larger masses through which cords and trabeculae of epithelial cells course. Often there will be a massive production of mucin which traps isolated cells and groups of cells diffusely. The mucin thus produced will in time become inspissated, contract and deform the epithelial cells contained within it. It is the presence of these peculiarly shaped, bizarre-looking cells that is so confusing in determining the benignancy of the tumor. Mitoses are frequently present. The inspissated mucin often has a chondroid appearance with a bluish matrix in which are seen isolated, contracted cells which are probably trapped epithelial cells, though it is considered possible that in rare instances fibroblasts included in such areas may undergo actual chondroid prosoplasia. Small areas of calcification are often seen in such areas, especially in very old, long-standing tumors. The very rare occurrence of bone in such tumors is explained by an osseous prosoplasia of fibroblastic elements. No incidence of osseous prosoplasia was encountered in this series.

The epithelial elements in the complex adenoma, in addition to forming typical ductal structures, solid sheets and reteform columns, often undergo squamous metaplasia. Islands of typical squamous cells which may produce whorls and strands of keratin are common in many of these adenomas. This keratin is said to provoke a rather marked fibroblastic response in certain areas of the tumor, especially at the periphery and may account for the dense, fibrous capsule which is sometimes seen. When such a fibroblastic reaction is seen within tht tumor itself it may lead to an erroneous diagnosis of sarcomatous change. Most pathologists now agree that such change does not occur in complex adenomas, though fibrosarcoma may very rarely arise as a primary tumor in a salivary gland as will be pointed out elsewhere in this article. Although there is often a dominant feature or pattern present in a given tumor, most, especially the larger ones, show a number of different areas representative of most of the varied patterns already described. The accompanying photomicrographs show the varied histologic appearance of these lesions (Figs. 4 through 16).

The gross features of the complex adenoma are nearly as variable as the microscopic features. The tumors are usually bulky and may be very large and pendulous, weighing as much as twenty pounds.[1] The surface is generally

[1] Cotterhill's case, reported by McFarland (1943). The tumor weighed 22 pounds.

FIG. 5. Patient with complex adenoma of parotid gland.

FIG. 6. Lateral view of patient with complex adenoma of parotid gland.

FIG. 7. The variation in the histological features of this complex adenoma show pseudocartilage at the upper portion of the field and very cellular epithelial elements in lower portion of field. X 78.

FIG. 8. An area of myxomatous tissue in a complex adenoma. Note sharp junction of neoplasm with salivary gland. X 170.

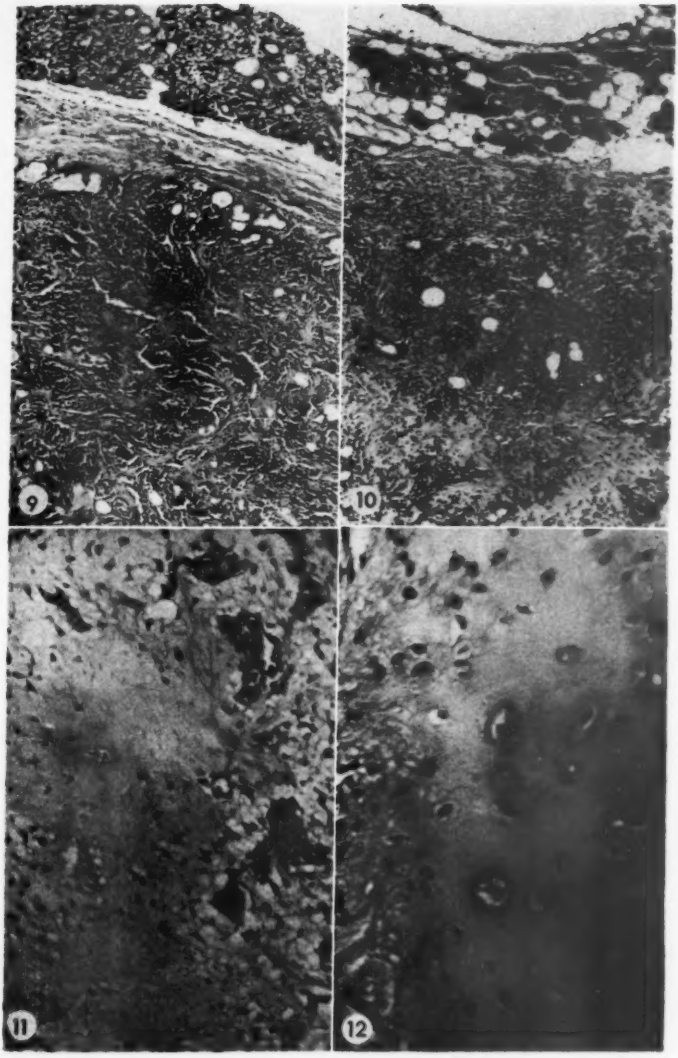


FIG. 9. Dense hyalin connective tissue associated with columns of epithelial cells. At the periphery of the complex adenoma is a rim of fibrous connective tissue. X 78.

FIG. 10. Duct-like spaces in this area of complex adenoma are associated with desmoplasia. X 78.

FIG. 11. Combination of clusters and single epithelial cells set in a myxochondroid tissue. X 170.

FIG. 12. An area of cartilage-like cells. X 270.

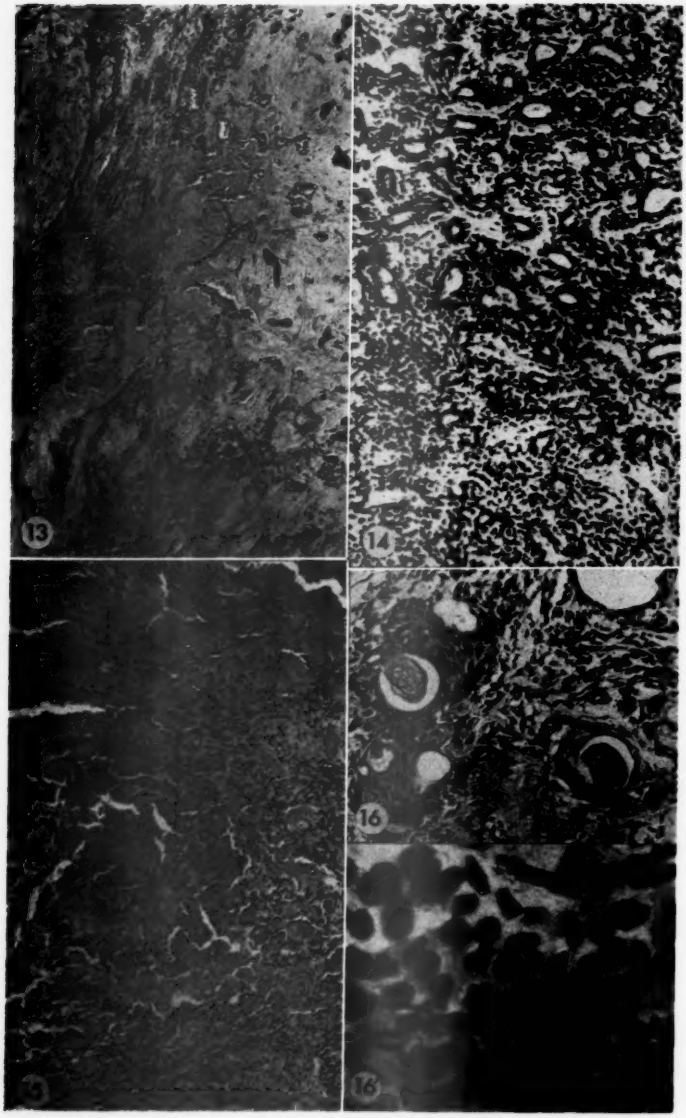


FIG. 13. Complex adenoma showing long columns of epithelial cells which are surrounded by fibrous connective tissue. X 78.

FIG. 14. Cellular area associated with ductal spaces. X 170.

FIG. 15. Marked fibrosis in complex adenoma. X 78.

FIG. 16. An area of squamous metaplasia in a complex adenoma. X 170.

FIG. 16'. A cellular area of a complex adenoma in which mitotic figures were prominent. X 730.

lobulated and surrounded by a tough, fibrous peripheral zone of varying thickness. However, there are often many small projections of adenoma which penetrate the substance of the gland. Occasionally there may be apparently separate tumor nodules within a parotid gland, though actually these discrete areas are connected by fine strands of tumor tissue. It is the presence of these projections and communicating strands that may give rise to recurrence, often many years later, when the tumor is merely shelled out, or small nodules are peeled out separately. This multi-nodular appearance is especially common in recurrent adenomas, and has led many pathologists to speculate on a multicentric origin of these tumors. Clear-cut proof of such multicentric genesis is lacking, and when numerous small tumor nodules recur in a salivary gland, even after a lapse of many years, it is probably because many small projections or strands of the original tumor were left in the gland when the main mass was "shelled out."

Clinically these tumors are very slow-growing, painless and consequently may be present for a number of years before the patient presents himself for excision of the rather bulky lesion. The tumors commonly begin in the fourth and fifth decades, though one patient in this series was an eleven year old boy and three were under twenty when first operated on. The average age at which the patients in this group was treated was 44.6 years and the average duration of the tumor at the time of treatment was 7.1 years. One patient had her tumor 35 years before seeking surgical treatment. It is usually stated that these complex adenomas occur more frequently in women, and our experience tended to bear this out. Of 119 such tumors which we studied, 70 were in females and 46 were in males. There were 3 cases studied histologically in which the sex of the patient was not recorded.

Other than age and sex no predisposing factors are definitely known to be of significance in regard to complex adenomas. Marsden (1951) has made an interesting study of these neoplasms in Malaya and has reported them to be more common among Malays and Chinese than among either Europeans or natives of India. Of some interest also is his finding that about 20 per cent of Malays and about 10 per cent of Chinese show some degree of salivary gland enlargement resulting from malnutrition. Trauma, infection and stasis resulting from obstruction from calculi have all been implicated as possible etiologic factors, but in actual fact there is little definite knowledge regarding the role of such conditions in the production of these tumors.

The very high incidence of recurrence which is usually reported following excision of these tumors is now generally accepted to be the result of inadequate initial excision. Most surgeons who have dealt with these tumors have remarked the ease with which they can be shelled out of their bed in the salivary gland tissue. Careful histologic examination of many glands totally removed with the tumor in situ has proved the existence of minute projections of tumor into normal gland beyond the apparent gross line of cleavage. For many years it was thought that some histopathologic correlation between these adenomas and their likelihood of recurrence could be found. McFarland (1942) has perhaps most accurately stated the complete lack of any correlation between histologic structure and prognosis. As a result of pathological investigation along these lines and as a result of his long-standing interest

in these tumors, the late A. O. Singleton (1943) concluded that cure depended entirely upon adequate local excision. He adopted a more radical approach to the management of these lesions which included sacrifice of the facial nerve in many instances followed by immediate plastic repair utilizing fascial slings. As a result of this approach a very low recurrence rate has been obtained at this institution. With the demonstration that total parotidectomy is possible without section of the major branches of the VIIth Cranial nerve the Singleton approach has been modified and parotid gland adenomas are either widely excised after preliminary exposure and identification of the nerves by means of electrical stimulation or total parotidectomy with preservation of the nerve is done. Of the tumors operated on at this hospital 21 were known to be recurrences following previous excision elsewhere. In 47 patients followed for an average of 8 years who had been operated on here, the recurrence rate was 17 per cent. Singleton (1942) reported a recurrence rate of only 8.7 per cent in his cases, some of which are included in the present report.

Adenoma with Lymphoid Stroma. These are the rare, highly distinctive tumors designated as papillary cystadenoma lymphomatosum and first described by Albrecht and Artz in 1910. The eponymic designation of Warthin's tumor has resulted from A. S. Warthin's report, in 1929, of two such cases with a rather classic description of the histopathology. To date about two hundred such cases have been reported in the literature, the largest group having been reported by Foote and Frazell (1953) who exained 44 of these tumors at Memorial Hospital. Thompson and Bryant (1950) have contributed 23 cases to the rapidly growing literature. There were six examples of this tumor in our series. The average age was 54 years, and all were located in the parotid gland. According to Foote these tumors are about eight times as common in males as in females and of the two hundred cases thus far reported all have occurred in the area of the parotid gland. This latter fact, together with the tendency for these tumors to occur later in life (fifth and sixth decades) lends support to the views of Thompson and Bryant regarding their histogenesis. These authors conclude that this neoplasm is related to a proliferation of parotid ducts included in lymph nodes. Neisse found such lymphoid tissue in intimate association with salivary tissue in the parotid gland. It is reported to be especially common in the lower pole of the superficial lobe, and Andrew (1952), in an anatomical study, found lymphoid tissue to be more abundant in the glands of elderly people. We agree with the majority of writers who now consider the papillary hyperplasia of the ductal epithelium to be the essential process involved in this neoplasm. The presence of the lymphoid tissue and follicles is incidental to the anatomical location of the tumor and is not primarily involved in the neoplastic growth. We have observed papillary changes identical to those seen in this type of adenoma occurring in salivary ducts within the lymphoid tissue adjacent to the lower pole of the parotid gland. Such papillary changes may also occur in the ductal epithelium elsewhere in the gland and without any associated lymphoid tissue. Conclusive proof of malignant change has never been reported in a typical papillary cystadenoma lymphomatosum, though Gaston and Tedeschi (1946)

report a recurrent lympho-epithelial tumor which they thought might have represented a malignant degeneration of a benign "adenolymphoma." It would seem to be a reasonable assumption that such a change may very rarely occur in a benign papillary cystodenoma lymphomatosum, though conclusive proof of this is lacking.

The gross appearance of the usual adenoma with lymphoid stroma is rather characteristic. Like the oxyphilic cell adenoma, they are small, spherical or ovoid in shape and rarely exceed 5 or 6 cm. in their greatest diameter. They are also fairly well defined tumors as a rule. They are unlike the solid, oxyphilic cell adenomas, however, in that they are characteristically cystic, with small compartments filled with brownish fluid or mucoid material. The cut surface usually bulges and is slimy in appearance, with grey-white mottled areas of lymphoid tissue contrasting to the rather homogeneous, pinkish-brown fleshy appearance of the oxyphilic cell adenoma. The cystic compartments are seen to be lined by shaggy papillary folds of epithelium.

Although the gross features are usually sufficiently distinctive to be diagnostic, it is in the microscopic appearance that these tumors are most remarkable. There are numerous papillary folds of large cuboidal cells in a double layer (Figs. 17, 18, and 19). These cells have well marked cell membranes and a basement membrane which seems to follow the papillary folds. The cytoplasm is brightly acidophilic and usually slightly granular. The presence of the lymphoid tissue lends the really distinctive feature to these tumors. It is variabe in amount, but typically occurs in well-defined follicles with definite germinal centers. The mature lymphocytes at the periphery of the clusters or follicles lie in contact with the basement membrane of the epithelial cells. There is some controversy regarding the exact role of this lymphoid tissue. It does seem to be increased in actual mass above that normally seen in this location. On the other hand it is not a neoplastic or atypical hyperplasia, and probably simply represents a reactive hyperplasia of the type often seen in areas adjacent to cystic or necrotic tissue.

The clinical features of the adenoma with lymphoid stroma almost always suggest their essential benignancy. They tend to remain small, or to enlarge very slowly and almost imperceptibly over a period of years. Occasionally they may enlarge rather rapidly possibly due to hemorrhage or accumulation of fluid. They are usually diagnosed as complex adenomas because of their location, slow, painless growth and nodular appearance. A very fluctuant or cystic tumor at the lower pole of the parotid may well be mistakenly diagnosed as branchial cyst. Although simple excision is usually thought to be curative, Frazell (1953) reports that there were six recurrences following forty-nine surgical procedures done for these tumors. Large numbers of cases are not available for follow-up to determine the true recurrence rate, however the report from Memorial Hospital would seem to indicate the necessity for adequate local excision including a margin of normal salivary gland tissue. Lederman (1943) reported the radiation treatment of two cases of recurrent "adenolymphomas" with good results.

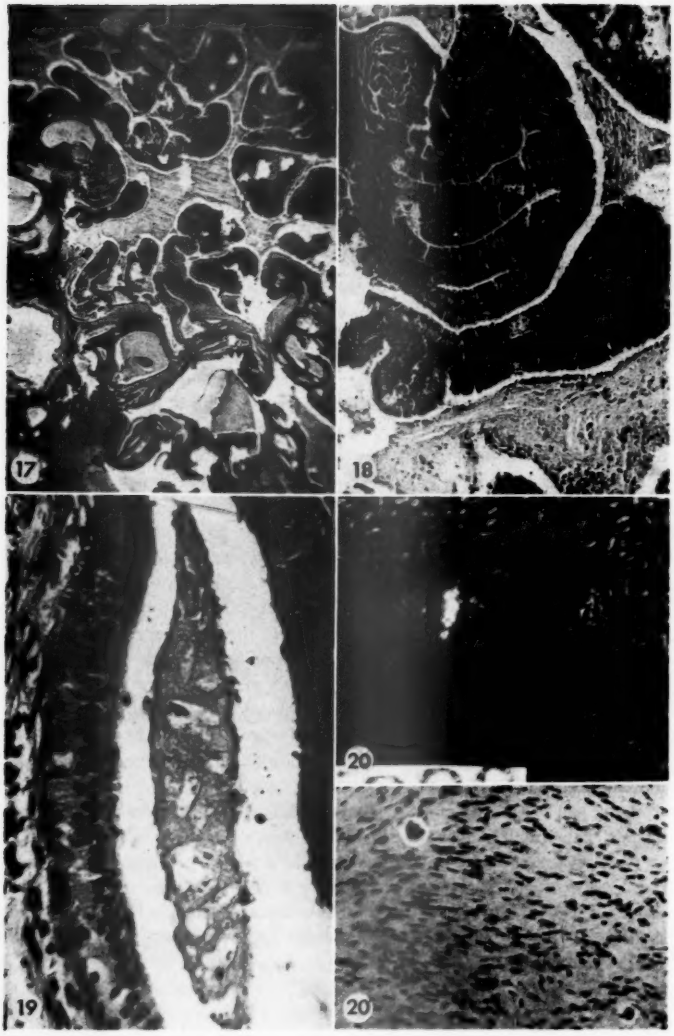


FIG. 17. Papillary cystadenoma lymphomatosum showing papillary projections of epithelial cells associated with lymphoid tissue. X 22.

FIG. 18. Distinct projections of epithelial cells into spaces filled with secretory material. X 270.

FIG. 19. Double layer of nuclei of epithelial cells which form papillary projections (Warthin tumor). X 270.

FIG. 20. Gross photograph of a well defined, soft, grayish white neurilemoma which has a glistening cut surface.

FIG. 20'. A neurilemoma arising from the facial nerve and occurring in the region of parotid gland neoplasms. X 170.

Carcinomas

With the exception of the muco-epidermoid carcinomas the histopathology and clinical behavior of salivary gland carcinoma differs little from that of glandular carcinoma in other organs. The majority of these malignant neoplasms arise from the ductal epithelium just as do the adenomas. There can be little doubt, moreover, that certain of the adenomata, if left in situ long enough, will become malignant. But the great majority of such malignancies appear to arise as a primary neoplastic process and to follow a fairly predictable clinical course depending upon their individual characteristics. Stewart and Foote (1945) have reported a group of fairly common malignant tumors peculiar to the salivary glands and these are designated as muco-epidermoid carcinomas. Because of their distinctive histological features and their relatively uncertain clinico-pathological correlation, we will consider this group in some detail. There were sixteen instances of this tumor in this series, and clinical observations of interest in this group will be presented.

Mucoepidermoid Carcinoma. Stewart, Foote, and Becker (1945) have separated this histologically peculiar neoplasm from the complex adenomas with which it was usually classified in the past. The reason for classifying these tumors as a separate entity is based on the clinical behavior of the neoplasm as well as the morphologic appearance. The histopathology of the lesions in this group is sufficiently diversified to justify a designation of "malignant mixed tumor" or complex carcinoma. There are, however, certain histological features common to all tumors in this group and these will be discussed in some detail. As regards the clinical behavior of these tumors, considerable confusion exists as the result of previous attempts to subdivide them into two general groups: a "semi-malignant" variety which exhibits features of local invasiveness and a marked tendency to recurrence; and a highly aggressive form which has an order of malignancy comparable to the squamous cell carcinomas. Unfortunately the clinicopatholigic correlation of the tumors in these groups is not sharply defined, and among the sixteen mucoepidermoid tumors which we have studied in this series so few presented clear-cut histological features indicative of either marked or moderate malignancy that we did not feel justified in further subdividing them. Therefore, we agree with Ackerman that all tumors of this histological type should be regarded as malignant, and the thoroughness with which their surgical extirpation is carried out must be determined solely by clinical considerations.

The gross appearance of the mucoepidermoid tumor may suggest a complex adenoma in that a few have a fairly distinct fibrous periphery which appears to separate them from the normal gland tissue. The majority of these tumors, however, are scirrhous, poorly defined, locally infiltrating growths with no distinct plane of cleavage existing between the lesion and the normal gland. Even in those tumors which appear at operation to have a fairly well-defined capsule, microscopic evidence of capsular invasion can nearly always be found, therefore resection of surrounding tissue is indicated.

Microscopically the mucoepidermoid carcinoma may present considerable difficulty in diagnosis because of the inconstancy of the cellular pattern. Only

two features seem to be common to all of these lesions, and they are extremely variable in degree. The first feature is that of mucin-production (Fig. 21). It is well known that the epithelial cells of the salivary ducts may secrete mucin under certain conditions. In the complexe adenoma, however, the mucin which is present has been subjected to certain chemical and physical alterations which gives it a different appearance and histochemical reaction. Moreover, the epithelial cells are not seen to be actively secreting mucin as is the case with the muco-epidermoid tumor. In the latter case, large epithelial cells bulging with mucin are often seen scattered through the tumor or found in clusters alternating with the typical squamous elements which form the second distinctive feature of the tumor. In places, small "lakes" of epithelial mucus may be seen surrounded by epithelial cells. The second element which must be seen in these tumors before the diagnosis of mucoepidermoid can be made is that of typical squamous epithelium (Fig. 22). This, again, may be present in small areas in the typical complex adenoma as a result of squamous metaplasia of the ductal epithelium. In the mucoepidermoid tumor, however, it is a prominent feature and often shows features of de-differentiation and mitotic figures which indicate a more active process of growth. Whorls of keratin are fairly common as is individual cell keratinization. According to Foote (1953) these tumors reproduce their original histological features (including mucin-production) in the metastatic deposits. Unfortunately we have not had the opportunity to study post-mortem material from a patient with metastatic disease. Foote also states that in the more aggressive and actively metastasizing of these tumors there are increasing proportions of squamous epithelium and fewer mucin producing cells. This would seem to indicate that some correlation exists between the histological appearance of the tumor and the clinical prognosis. In the rather small number of tumors of this type which we have seen, however, this correlation did not occur often enough to be of statistical significance in determining the prognosis.

The mucoepidermoid carcinoma is a fairly common malignancy. Stewart and Foote (1945) found 45 cases among the 700 salivary gland tumors which they originally studied, an incidence of 6.4 per cent. The 16 cases in the series here reported represents an incidence of 8.9 per cent. The average age of patients in this series was 42 years, which is slightly younger than in the complex adenoma group. Two patients in this group were aged 23 and 24 respectively. Both were subjected to total parotidectomy with sacrifice of the facial nerve and there have been no recurrences or metastases 4 and 12 years postoperatively. It is also of interest to note that 12 of our patients were males and only 4 were females. This is in contrast to the marked preponderance of females in the complex adenoma group. Other features observed in this group of tumors which might lead to a clinical suspicion of the malignant nature of the condition included pain, fixation of the overlying skin to the tumor, and in some instances a stony hard consistency which was never present in the complex adenomas. Facial nerve palsy was not seen in any of these patients as a preoperative sympton. Of nine patients followed from one to six years after operation only two are known to have had recurrences and metastases. One patient died of the disease six years after treatment, and the second, who had widespread metastases when he was first seen, died six months later. No

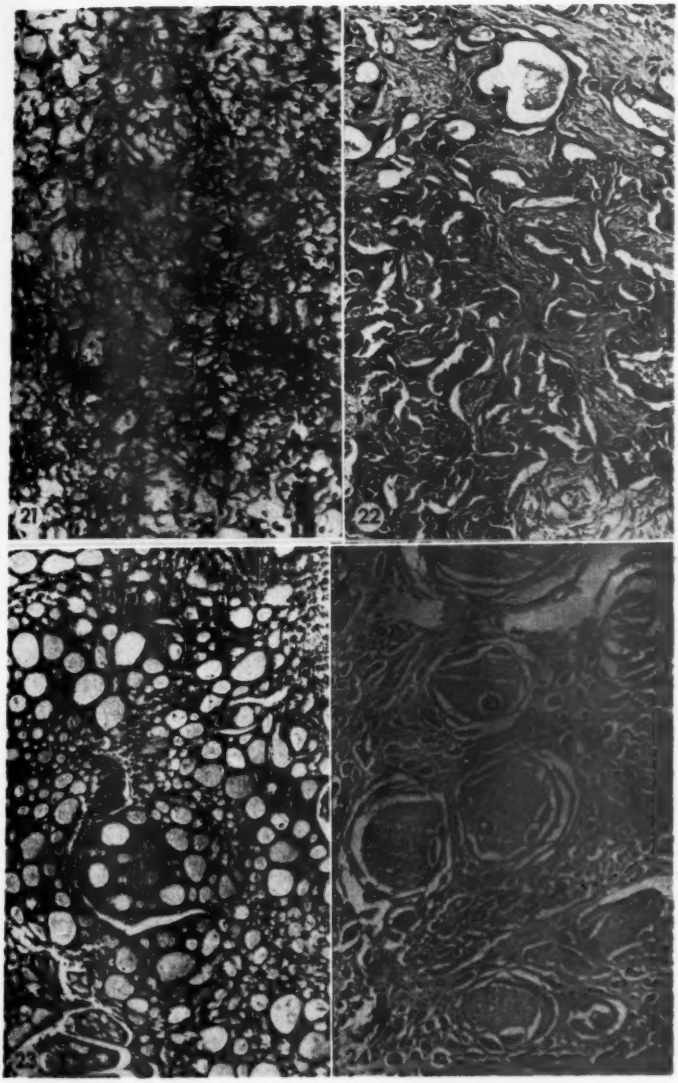


FIG. 21. Moderately well differentiated mucoepidermoid carcinoma which shows areas in which acinar spaces are formed by mucin secreting epithelial cells. X 170.

FIG. 22. An area of less well differentiated squamous cells from a mucoepidermoid carcinoma. X 170.

FIG. 23. Typical pattern encountered in cylindroma which simulates an adenoid cystic carcinoma. X 78.

FIG. 24. Perineural lymphatic involvement by cylindroma. X 78.

definitive surgical procedure was carried out in this latter instance. From our experience with this group of cases we can concur with Kirklin, McDonald, Harrington and New (1951) that the mucoepidermoid tumor is a malignant neoplasm but one with a considerably more favorable prognosis that the types of carcinoma to be dicussed in succeeding paragraphs,

Adenocarcinoma. The adenocarcinomas studied in this series represented 11.9 per cent of the total group of salivary gland neoplasms. Most adenocarcinomas arise from the ductal epithelium and vary from the well-differentiated cylindroma type to very poorly differentiated, highly malignant forms. Two examples of acinic cell adenocarcinoma described by Buxton (1953) and Godwin and Foote (1954) were present in this group.

The cylindroma type of adenocarcinoma has been reported to be a fairly common form. Kirklin, *et al.*, (1951) report an incidence of 4.7 per cent in 717 tumors of the parotid salivary gland. Of the twenty-two cases of adenocarcinoma in this series only three were diagnosed as cylindroma, an incidence of 1.7 per cent of the entire group of neoplasms studied. Two of these tumors occurred in the submaxillary gland and one the parotid glad. One case occurred in a 29 year old female who had cervical metatases but is still alive and apparently well nine years after excision and radical neck dissection. The second case of cylindroma of the submaxillary gland was in a 50 year old man who is alive two years post-operatively but has roetgenologic evidence of pulmonary metastases. The third case, which occurred in the parotid gland, could not be traced.

The small number of patients which we have observed with this tumor is perhaps due to the fact that very rigid criteria were employed in the histological diagnosis of this lesion and only those showing the classical pattern described by Ackerman (1953) were accepted by us.

The gross features of the cylindromatous type of adenocarcinoma do not differ significantly from other adenocarcinomata. They are firm, greyish-white in color and are fixed to surrounding structures, often including the facial nerve. The classical cylindroma has a very distinctive microscopic appearance which is characterized chiefly by a well-defined glandular pattern with production of rather large amounts of mucin (Fig. 23). This substance, when seen within the gland space, often has a rather foamy, basophillic-staining appearance. The epithelial cells are often quite well differentiated and may give rise to a false impression of benignancy. Also the fact that strands or cords of the epithelial cells may be seen surrounded by a mucinous stroma occasionally leads to an erroneous diagnosis of "mixed tumor," or perhaps of "malignant mixed tumor." Careful microscopic study, however, will reveal highly infiltrative tumor cells in adjacent structures, in lymphatic spaces and especially in the perineural lymphatics for which these tumors seem to have a special affinity (Fig. 24). Buxton (1953) has proposed the term "pseudoadenomatous basal cell carcinoma" for these neoplasms. This is a rather apt descriptive term, however, as he points out, it is an unwieldy term and one which may lure the surgeon into a false sense of security regarding the prognosis. These tumors are well known to pursue a slow but relentless course, and a very high percentage of them will have cervical lymph node

metastases. Pulmonary and other visceral metastases have also been reported rather frequently from these neoplasms. It is now generally accepted that these tumors should be treated by radical excision and cervical node dissection.

Of the remaining 18 cases of adenocarcinoma included in this series, two were of the acinic cell type which has recently been described in some detail by Godwin, Foote, and Frazell (1954). This type is considered to be rare, is slow-growing, often rather large and fairly well encapsulated. Both acinic cell adenocarcinomas occurred in the parotid gland. They may easily be mistaken grossly and clinically for complex adenomas, and, as they seldom metastasize, may usually be treated by sub-total parotidectomy or total parotidectomy with preservation of the facial nerve. Neither of our patients with these tumors has had a recurrence 2½ and 6 years following the initial excision. Because of the relative scarcity of these acinic cell adenocarcinomas a more detailed discussion of them cannot come within the scope of this paper. Representative microscopic fields are shown in figure 25.

There was one unusual adenocarcinoma of a papillary type which presented a pattern of well-differentiated papillary folds of ductal-type epithelium projecting into small cystic spaces and invading the adjacent tissues. This tumor occurred in a 30 year old male physician and was treated by widespread local excision with preservation of the facial nerve. A letter from this patient states that he has had no recurrence or evidence of metastasis 13 years post-operatively, though a small lipoma was removed from the same area about two years following the original excision (Fig. 26).

Of some interest was the occurrence of a well-differentiated adenocarcinoma in a large complex adenoma which arose in the soft palate of a 79 year old man. This tumor had been partially excised several years previously, but had recurred and had recently showed rather rapid growth with invasion of the right maxillary sinus and orbit. The tumor was successfully removed by en block resection and the patient has no evidence of recurrence six months post-operatively. Lambert (1952) has studied 39 salivary gland tumors arising in the hard and soft palate and found 20 of them to be malignant. He concluded that tumors arising in salivary gland tissue in these locations are much more likely to prove to be malignant that those arising in the major salivary glands.

Of the adenocarcinomas remaining in this group, all were considered to be of ductal origin and all but one arose in the parotid gland. Although microscopically showing various degrees of differentiation, all were of a rather high order of clinical malignancy. Of eleven patients who could be traced, none is alive and free of cancer. Table 3 shows the course of these adenocarcinomas and bears out the poor prognosis which these tumors are generally stated to have. Little correlation could be found between the histological differentiation of these neoplasms and the rapidity with which they grow and metastasize. Grossly these carcinomas are firm or even stony hard, they are not encapsulated and are frequently fixed to skin and deeper structures. Microscopically they may show fairly well differentiated ductal structures or form gland-like spaces or they may be quite poorly differentiated with cords and strands of tumor cells which show numerous mitotic figures and lymph vascular invasion (see Figs. 26′ and 27).

Clinically these tumors pose little difficulty in diagnosis (Figs. 28 and 28′). They are of rather rapid growth, have a peak incidence at around fifty years of age (51.1 years average in this series), show no predilection for either sex, and are almost always associated with pain. In the more advanced cases there may be facial nerve palsy and bone invasion or metastases can often be demonstrated by X-ray. These adenocarcinomas spread chiefly by lymphatic

TABLE 3

ADENOCARCINOMAS

Path. No.	Sex	Age	Site*	Duration	Follow-Up
			A. *Acinic Cell Adenocarcinomas*		
S-9556	M	47	P	1 month	N. K. R. 2½ yrs. post-op.†
S-16247 ...	M	64	P	5 years	L. W. M. (cervical) 6 yrs. post-op.‡
			B. *Cylindromas*		
S-15333 ...	F	29	S	9 years	N. K. R. 4 yrs. post-op.
S-31321 ...	M	69	S	5 years	L. W. M. (pulmonary) 2 yrs. post-op.
D-24201 ...	F	27	P	?	No clinical data available.
			C. *Other Adenocarcinomas*		
D-68	?	?	?	?	No clinical data available.
D-24610 ...	M	49	P	?	No clinical data available.
S-32	F	31	P	1 year	N. K. R. 10 yrs. post-op.
S-1652	M	59	P	10 mos.	L. W. M. (cervical) 1 yr. post-op.
S-1874	M	38	P	8 mos.	D. W. M. 2 yrs. post-op.§
S-3101	M	?	?	?	No clinical data available.
S-4911	M	32	P	1 year	D. W. M. 2 yrs. post-op.
S-10155 ...	M	81	P	6 mos.	D. W. M. 18 months post-op.
S-11587 ...	M	30	P	2 mos.	N. K. R. 13 yrs. post-op.
S-17200 ...	F	56	P	21 yrs.	L. W. M. 1 yr. post-op.
S-19502 ...	F	24	P	1 year	D. W. M. 14 months post-op.
S-19590 ...	M	61	P	1 year	Lost to follow-up
S-20402 ...	M	60	P	18 mos.	D. W. M. 1 yr. post-op.
S-25408 ...	F	64	P	5 yrs.	L. W. M. 8 yrs. post-op.
S-42155 ...	F	41	S	2 yrs.	N. K. R. 4 mos. post-op.
S-43937 ...	M	79	palate	16 mos.	N. K. R. 6 mos. post-op.

* Site: P Parotid; S submaxillary.
† N. K. R. No known recurrences.
‡ L. W. M. Living with metastases.
§ D. W. M. Dead with metastases.

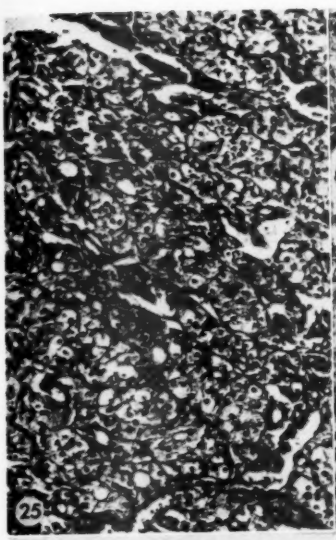
FIG. 25. Acinic cell adenocarcinoma with cords and nests of large cells presenting vaculoization of cytoplasm. These are low grade adenocarcinomas. X 170.

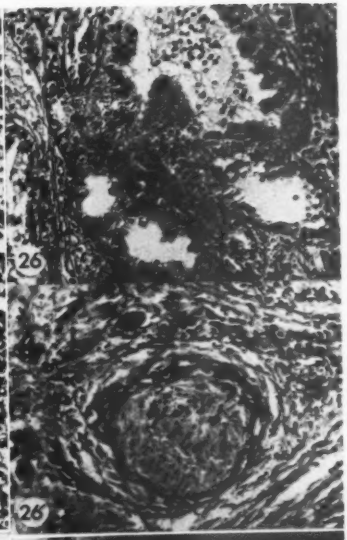
FIG. 26. Well differentiated adenocarcinoma showing papillary pattern. X 170.

FIG. 26′. Poorly differentiated adenocarcinoma of parotid gland with involvement of perineural lymphatics. X 170.

FIG. 27. A different area from the same neoplasm shown in Fig. 26′ which shows poorly formed acini and pleomorphic cells. X 170.

FIGS. 28 and 28′. Gross photograph of a patient with a lymphoepithelioma of the parotid gland.


25


26

27

28

28

metastases but blood borne metastases are not infrequently reported and distant spread to lungs and liver are fairly common. Treatment should include radical resection, probably accompanied by a unilateral neck dissection. Post-operative irradiation may be indicated in certain instances. Inoperable cases may show good initial response to radiation though it has not been shown to be curative.

Undifferentiated Carcinoma. In this group are included tumors so anaplastic that no definite indication as to their exact histologenesis could be determined. One might expect that the great majority of them are derived from ductal epithelium, but since this is impossible to prove they are placed in this category. Only four such cases were seen in this series, two in males and two in females. The average age of these patients was 62 years and there was a fatal outcome in each case, in some instances with wide-spread metastases. One case, however, which was discovered in the parotid of a man of 83, had only cervical metastases and an excellent response to X-ray was obtained. The man died four months later of unrelated causes, and at autopsy a few small carcinoma-containing lymph nodes in the lower anterior cervical chain were the only evidence of any residual malignancy.

Clinically these tumors are similar to adenocarcinoma of ductal origin, with more rapid growth and early metastases. Because of the fact that these tumors tend to occur in older people and inasmuch as they initially respond satisfactorily to X-ray surgery, other than for excision of tissue for biopsy, is seldom done.

Epidermoid Carcinomas. Primary squamous cell carcinoma arising in the salivary glands is of infrequent occurrence. Kirklin (1951) reports an incidence of 1.8 per cent in his large series of parotid tumors. There were eight histologically proven cases in this series (4.4 per cent). Squamous cell carcinoma secondarily involving the salivary glands is not infrequently seen, and this was eliminated in this study by careful review of the gross specimens, histological material and clinical records. We eliminated all cases of epidermoid carcinoma reported in the submaxillary gland because the frequency with which metastatic deposits occur in this region from lesions of the mouth and skin of the face actually makes it impossible to be certain in any instance that the tumor is primary in the submaxillary gland.

Epidermoid carcinoma arises in the salivary gland as a result of squamous metaplasia of the ductal epithelium. The fact that the patients in this group averaged 65 years of age suggests that this lesion is related to degenerative epithelial changes common to advanced age. The gross appearance is that of any infiltrating malignant neoplasms, and there may be ulceration of the overlying skin. Microscopically there are sheets and plugs of squamous epithelium exhibiting varying degrees of cellular differentiation.

Two patients in this group of epidermoid carcinomas had tumors classified as *lymphoepitheliomas.* Buxton (1953) considers these to be highly undifferentiated squamous cell carcinomas occurring in combination with mature lymphocytes. His report of five cases is the largest series that we could find in the literature, and most of the large series of salivary gland tumors thus far reported fail to mention this neoplasm. The presence of large masses of

mature lymphocytes alternating with sheets of very poorly differentiated epidermoid cells suggests that the neoplastic process is confined to the epithelial component, the lymphoid tissue merely participating as a reactive hyperplasia (Figs. 29, 30, 31, and 32). The clinical course and type of response to roentgen therapy reported by Buxton and confirmed in the two cases we have studied indicates that these tumors have the same biologic properties as poorly differentiated squamous cell cancers in general. They bear no resemblance to the so-called lymphoepitheliomas arising in the nasopharynx. In both of our cases careful and repeated otolaryngologic study, including excision of tissue for biopsy, failed to reveal any primary site in the nasopharynx. One of our patients, treated primarily by radical surgery, died three years later of generalized metastases. The second case is receiving roentgen therapy alone and thus far (2 months) the primary tumor mass has regressed in size, but there is radiological evidence of pulmonary metastases. The five cases reported by Buxton were treated by wide local excision (with radical neck disscetion in 3 cases) followed by post-operative irradiation. All were dead within a year. Buxton concluded that surgical treatment was probably not indicated for this form of malignancy. Lederman (1943) reported a case which was probably identical to these cases which he termed "malignant adenolymphoma" and which was treated primarily by X-ray (4000 R) with a good response. This patient survived for 10 years following treatment. On the basis of our own limited experience we do not feel justified in drawing any conclusions with regard to therapy in this lesion.

The clinical findings in epidermoid carcinoma of the parotid are essentially the same as in the adenocarcinoma group, with pain and facial palsy being somewhat more prominent. In the eight patients of this group, the duration of symptoms averaged less than a year, and all had a pre-operative clinical diagnosis of malignant neoplasm. In most instances the degree of anaplasia is well correlated with the clinical course, though factors such as age, general physical condition, and coexisting diseases undoubtedly influenced the outcome to a considerable extent. It seems logical on the basis of published reports as well as our own experience to conclude that surgical treatment is indicated in those cases of well differentiated squamous cell carcinoma thought clinically to be resectable (as determined by size, duration and local factors such as extension and lymphatic involvement) in which there is a reasonably good life expectancy after consideration of the patients general physical fitness. Highly undifferentiated lesions, clinically non-resectable cases, and poor risk patients in general are best treated by radiation alone. These tumors generally show early lymphatic spread, and if primary surgical treatment is elected it should include radical neck dissection.

Non-epithelial Neoplasms Arising in the Salivary Glands. Although the great majority of primary neoplasms of the salivary glands are of epithelial origin, it is obvious that isolated instances of tumors arising from the supporting structures of these glands will be encountered from time to time. These neoplasms may be of importance from the standpoint of differential diagnosis. Four such cases are included in the present study chiefly to indicate that, while rare, such tumors do occasionally occur. All of these lesions oc-

curred in children, but only one was malignant. There were two instances of benign hemangio-endothelioma occurring in female infants two and three months old. In both instances local excision has apparently resulted in cure. The microscopic appearance of these tumors is that of an invasive, non-encapsulated neoplasm which is attempting to form vascular spaces.

One case of neurilemmona has recently been encountered in a 13 year old girl and is reported here because of the apparent rarity of these tumors in the parotid. According to Bruni (1952) only six neurilemmonas have been reported (including his case) in this location. The gross and histological features of this tumor are illustrated in figures 20 and 20′. This is a benign lesion, but because of its occurrence in the sheath of major branches of the facial nerve its excision may result in some loss of function. When it occurs in an age group where cosmetic results are of the utmost importance it may assume considerable clinical significance.

There was one instance of sarcoma in this series. It occurred in the parotid of an 11 year old girl and was classified as a fibrosarcoma. Following surgical excision this tumor recurred and further treatment was denied the child by the parents. Death occurred about eight months later. Sarcoma, once a common diagnosis in salivary gland tumors, is now considered to be extremely rare. Most of the "fibromyxosarcomas" and related diagnoses were misapplied to complex adenomas. The subsequent benign course of the patients in whom these diagnoses were made has indicated the inappropriateness of these terms, and recent histological restudy of such tumors shows them to correspond to benign adenomas of epithelial origin previously described.

Discussion. For all practical purposes salivary gland adenomas can be considered as completely benign lesions of epithelial origin. Like adenomas arising elsewhere they occasionally eventuate in malignancy, though it is estimated (Ackerman) that less than five per cent of carcinomas occurring in these structures develop from a pre-existing adenoma. The term *complex adenoma* which Foote has proposed to replace the time-honored but meaningless term "mixed tumor" is used throughout this paper and as such connotes a benign neoplasm. This is by far the most common neoplasm arising from salivary gland tissue. The pathogenesis of these interesting tumors has been

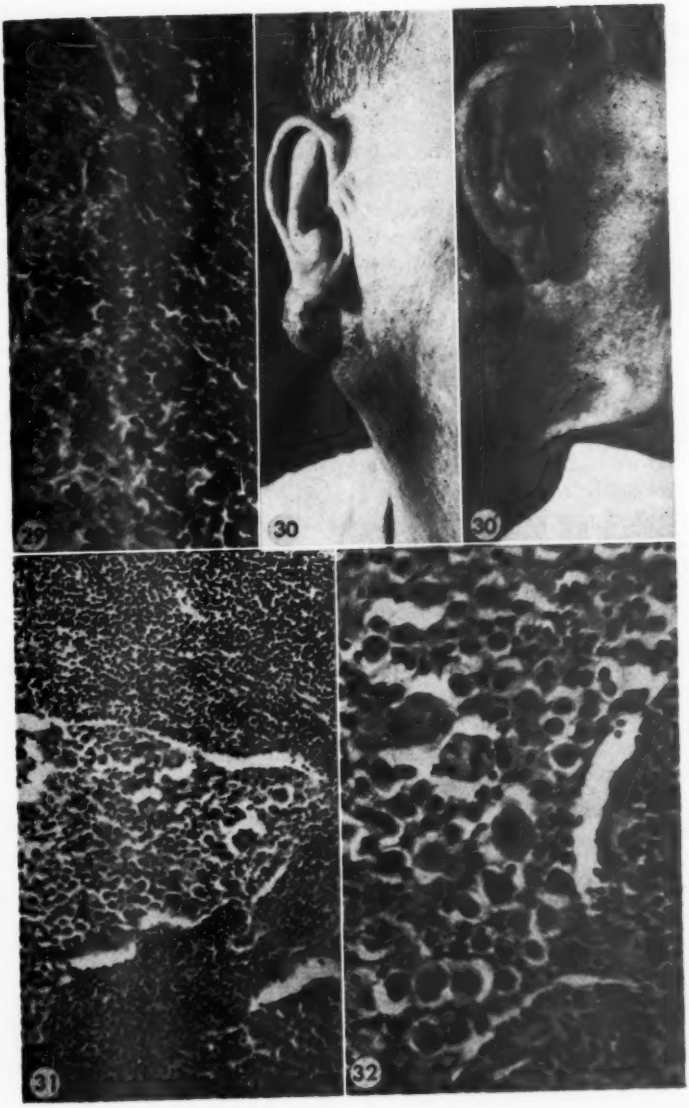
---

FIG. 29. Lymphoepithelioma of the parotid gland showing the intimate relationship of poorly differentiated epithelial cells and lymphocytes. X 270.

FIG. 30 and 30′. Clinical photograph of a patient with a lymphoepithelioma of parotid gland region before irradiation therapy (30) and after roentgen therapy (30′).

FIG. 31. Mixture of lymphocytes with neoplastic cells of a lymphoepithelioma. X 170.

FIG. 32. Pleomorphic cells in which there were numerous atypical mitotic figures. This photograph is from the same case as Fig. 31. X 270.


29
30
30
31
32

thoroughly elucidated and the origin from the epithelium of the salivary gland ducts is now generally accepted. These lesions are of clinical importance because of their well-known tendency to recur and because of their relation to the facial nerve when they occur in the parotid. The reason for such recurrence is generally thought to be due to inadequacy of the primary excision. Despite the deceptive appearance of complete encapsulation microscopic projections of tumor often extend beyond the apparent limits of the adenoma and into the normal gland tissue. Thus these tumors are "shelled out," as is so easily accomplished in most instances, multiple minute foci of tumor are left behind to develop slowly, often manifesting themselves many years later. The close proximity of the facial nerve to the parotid gland renders adequate excision of these tumors (especially when recurrent) difficult in this location. The much higher recurrence rate usually reported for complex adenomas arising in the parotid gland than in such tumors in other salivary glands is probably related to this fact.

Simple adenomas of either acinic cell or ductal cell origin are usually smaller than the complex adenomas, show fewer extensions beyond the apparent periphery and for this reason are less apt to recur following local excision. This is also true for the papillary cystadenoma lymphomatosum, though Foote has indicated that this latter tumor probably recurs more often than is usually supposed. These other adenomas are much less common than the complex adenomas and occur almost exclusively in the parotid gland. Because of their relatively small size (seldom exceeding 5 or 6 cm. in their greatest diameter) they are generally easy to remove with an adequate margin of normal tissue, no risk to the facial nerve being involved in the average case.

Carcinoma is seen in about twenty per cent of all tumors in the parotid gland and about thirty per cent of all tumors in the submaxillary gland. Lambert has reported an even higher incidence of malignancy in tumors arising in "ectopic foci" of salivary gland tissue. The biologic properties of salivary gland carcinomata vary from certain very slow-growing but locally invasive mucoepidermoid carcinomata to very rapidly growing, widely matastasizing undifferentiated carcinomas and ma-

lignant lymphoepitheliomas. Carcinomas arising in these glands are best classified in the four groups listed in table 1. These four main types can be further subdivided on basis of the histological features and each type tends to follow a fairly well recognized clinical course. Prognosis is well correlated to the type and the degree of differentiation of the carcinoma. Most carcinomas of salivary gland tissue are of the adenocarcinoma type and arise from the ductal epithelium. A rare form, the acinic cell adenocarcinoma, has been described by Buxton (1953) and reported in some detail by Godwin, Foote, and Frazell (1954). This unique carcinoma is thought to arise, at least in some instances, from a pre-existing acinic cell adenoma, and tends to follow a rather benign course. Two such tumors were encountered in the present series.

Carcinoma arising in the parotid gland usually demands a radical surgical attack, and preservation of the facial nerve should not be considered except in certain of the well differentiated examples of mucoepidermoid carcinoma and in the acinic cell adenocarcinomas. Radical neck dissection probably should accompany the operation for cylindroma and epidermoid carcinomas in general. In the undifferentiated carcinomas and malignant lymphoepitheliomas there is reason to believe that radiation therapy may be preferable in most instances to a surgical attack.

Most primary carcinomas of the submaxillary gland are of the cylindroma type, and this location is more favorable to radical en block resection and node dissection than is the parotid gland area. Post-operative irradiation may be indicated in the more advanced cases, especially where the histological diagnosis indicates a fair degree of radiosensitivity.

Summary

One hundred and eighty tumors of the salivary glands seen in the Department of Pathology and the Laboratory of Surgical Pathology at The University of Texas Medical Branch have been subjected to careful and critical histologic review. In many instances the original diagnosis was changed to conform with modern terminology. These tumors were classified according to a simple and logical classification which clearly indicates the

benign or malignant nature of the tumors. The histogenesis of each type of neoplasm is discussed and correlated with known clinical data. Photographs of important clinical and microscopic features are presented as an aid to diagnosis. Certain conclusions with regard to the type of treatment indicated in each instance could be drawn on the basis of follow-up studies conducted by the Tumor Clinic of this Institution. A few rare tumors not previously reported are included and discussed in some detail in this presentation. The recent revival of interest in the pathology of the salivary gland tumors combined with newer techniques in surgical and radiological treatment should result in a lower morbidity rate for the benign group of lesions and a lower mortality rate and increased long-term survivals in the carcinoma group.

Acknowledgment

Doctor Robert M. Moore, Professor of Surgery, The University of Texas Medical Branch, assisted in preparation of the manuscript and permitted the inclusion of his cases in this report. Other members of the University staff that have been of great assistance in preparing this material include Doctor E. J. Poth, Professor of Surgery; Doctor T. G. Blocker, Professor of Plastic and Maxillofacial Surgery; Doctor A. O. Singleton, Jr., Associate Professor of Surgery, and other members of the staff.

REFERENCES

Ackerman, L. V.: Oncocytoma of the Parotid Gland. *Arch. Path.*, 36: 5–8–511, 1943. *Surgical Pathology*, 358–376. C. V. Mosby Co., St. Louis, 1953.

Andrew, W.: A Comparison of Age Changes in Salivary Glands of Man and of the Rat. *J. Gerontology*, 7: 178–190, 1952.

Benedict, E. G., and J. V. Meigs: Tumors of the Parotid Gland; A Study of 225 Cases with Complete End Results in 80 Cases. *Surg. Gyn. Obs.*, 51: 626–647, 1930.

Bruni, C.: Morbid Anatomical Studies on Tumors of the Parotid Region. X Neurilemonas. *Lav. Inst. Anat. Istol. Patol. Univ. Perugio*, 12: 257–269, 1952.

Buxton, R. W., J. H. Maxwell, and D. R. Cooper: Tumors of the Parotid Gland. *Laryngoscope*, 59: 565–594, 1949.

Buxton, R. W., J. H. Maxwell, and A. J. French: Surgical Treatment of Epithelial Tumor of the Parotid Gland. *Surg. Gyn. Obs.*, 97: 401–416, 1953.

Foote, F. W., Jr., and E. L. Frazell: Tumors of the Major Salivary Glands. *Cancer*, 6: 1065–1133, 1953.

Fry, R. M.: The Structure and Origin of the Mixed Tumors of the Salivary Glands. *Brit. J. Surg.*, 15: 291–306, 1927.

Gaston, E. A., and C. G. Tedeschi: Adenolymphoma of the Parotid and Submaxillary Glands. *Amer. Surg.*, 123: 1075–1089, 1946.

Godwin, J. T., and S. H. Calvin, Jr.: Adenoma of the Parotid Gland. *Arch. Path.*, 46: 187–189, 1948.

Godwin, J. T., F. W. Foote, Jr., and E. L. Frazell: Acinic Cell Adenocarcinoma of the Parotid Gland. Report of Twenty-seven Cases (with eleven illustrations). *Am. J. Path.*, 30: 465–478, 1954.

Harris, P. N.: Adenoma of the Salivary Glands. *Am. J. Cancer*, 27: 690–700, 1936.

Hoover, W. B.: Submaxillary Gland Tumors. *Surg. Clin. N. Am.*, Lahey Clinic Number, 671–675, 1953.

Kirklin, J. W., J. R. McDonald, S. W. Harrington, and Gordon B. New: Parotid Tumors; Histopathology, Clinical Behavior, and End Results. *Surg. Gyn. Obs.*, 92: 721–734, 1951.

Lambert, V.: Mixed Salivary Tumors of the Upper Respiratory Tract. *Proc. R. Soc. Med.*, 45: 826–828, 1952.

Lederman, M.: Adenolymphoma of the Parotid Salivary Gland. *Brit. J. Rad.*, 16: 382–385, 1943.

Marsden, A. T. H.: The Distinctive Features of the Tumors of the Salivary Glands in Malaya. *Brit. J. Cancer*, 5: 375–381, 1951.

Marshall, S. F., and G. O. Miles: Tumors of the Submaxillary and Parotid Gland Areas. *Surg. Clin. N. Am.*, 27: 501–516, 1947.

Martin, Hayes, and H. E. Ehrlich: Papillary Cystadenoma Lymphomatosum of the Parotid Salivary Gland. *Surg. Gyn. Obs.*, 79: 611–623, 1944.

McFarland, Joseph: Three Hundred Mixed Tumors of the Salivary Glands of Which Sixty-nine Recurred. *Surg. Gyn. Obs.*, 63: 457–468, 1936. The Mysterious Mixed Tumors of the Salivary Glands. *Surg. Gyn. Obs.*, 76: 23–34, 1943. The Histopathologic Prognosis of Salivary Gland Mixed Tumors. *Am. J. Med. Sc.*, 203: 502–519, 1942.

McKnight, H. A.: Malignant Parotid Tumors in the Newborn. *Am. J. Surg.*, 45: 128–130, 1939.

Meza-Chavez, Luis: Oxyphilic Granular Cell Adenoma of the Parotid Gland (Oncocytoma); Report of Five Cases and a Study of Oxyphilic Granular Cells (Oncoytes) in Normal Parotid Glands. *Am. J. Path.*, 25: 523–548, 1949. Sebaceous Glands in Normal and Neoplastic Parotid Glands. *Am. J. Path.* 25: 627–645, 1949.

Quattlebaum, F. W., M. B. Dockerty, and C. W. Mayo: Adenocarcinoma, Cylindroma Type, of the Parotid Gland; A Clinical and Pathologic Study of Twenty-one Cases. *Surg. Gyn. Obs.*, 82: 342–347, 1946.

Rawson, A. J., J. M. Howard, H. P. Royster, and R. C. Horn, Jr.: Tumors of the Salivary Glands; A Clinicopathological Study of 160 Cases. *Cancer*, 3: 445–458, 1950.

Singleton, A. O., and N. Duren: Tumors of the Salivary Glands. *Tex. State J. Med.*, 36: 784–792, 1941.

Singleton, A. O.: Tumors of Salivary Glands, Benign and Locally Malignant. *Surg. Gyn. Obs.*, 74: 569–572, 1942.

Stewart, F. W., F. W. Foote, and W. F. Becker: Muco-epidermoid Tumors of the Salivary Glands. *Ann. Surg.*, 122: 820–844, 1945.

Swinton, N. W., and S. Warren: Salivary Gland Tumors. *Surg. Gyn. Obs.* 67: 424–435, 1938.

Thompson, A. S., and H. C. Bryant, Jr.: Histogenesis of Papillary Cystadenoma Lymphomatosum (Warthin's Tumor) of the Parotid Salivary Gland. *Am. J. Path.*, 26: 807–849, 1950.

Willis, R. A.: *The Pathology of Tumors*, 320–348; Butterworth & Co., Ltd. London, 1948.

AUTOINTOXICATIONS AND PROCESSES OF AGEING

V. KORENCHEVSKY*

There are the primary basic causes of ageing, about which so far nothing is known, while some knowledge is available about numerous secondary causes. Although the latter are operating only in some cases of old age, the acceleration and aggravation of ageing, produced by them in these cases, are often very pronounced. Therefore the secondary causes, separately or in groups, were claimed by some authors to be responsible for the whole process of ageing. These theories of ageing are purely hopothetical, and nearly all of them have no experimental foundation.

Some of the causes of ageing appear to *operate during the whole life of all individuals*, being to a certain extent made harmless in young and adult ages by existing neutralising mechanisms. Apparently, since with ageing the action of these causes becomes prolonged, their cumulative effects start to accelerate and aggravate the processes of ageing. In this way these agents become one of the important causative factors of premature senescence and senility.

The same effects should be manifested at earlier ages of life when, for some pathological reason, these causes become increased in their severity, or when the neutralising mechanisms prematurely weaken.

Probably this group of permanently active causes of ageing might be placed on the borderline between the primary and secondary causes of old age. The autointoxications with metabolic and gastro-intestinal products should belong to this "borderline" group. There are many investigations which suggest the theory that autointoxications might cause some ageing effects. Most of these investigations, however, are of an indirect nature and deficient in various ways, being devised in most cases without any relation to the problem of ageing, or in some cases performed with an unsatisfactory technique.

---

* From the Gerontological Research Laboratory, Whittington Hospital, London. Received for publication June 11, 1954. Reference numbers are omitted from the text by editorial direction.

It is surprising that there is no study in which all those available data are collected and analysed. The present paper attempts to fill this gap.

Ageing Effect of Overfeeding

It is obvious that when large quantities of food are consumed, metabolic waste products are produced in greater quantities and, therefore, might accumulate in the organs, tissues and fluids of the organism. Experiments on animals and observations on human beings appear to prove that overfeeding shortens the span of life and produces some other damaging effects. Thus, Rudzinska on the infusorium Tokophrya, Ingle on crustacea Daphnia longispina and Moina macrocopa, McCay *et al.*, on brook trout, Carrel, Vischer *et al.*, White *et al.*, and Tannenbaum on mice, McCay *et al.*, and Carlson and Hoelzel on rats found that diets restricted in calories, or intermittent fasting prolong the span of life, and maintain or improve fertility; while overfeeding shortens life and impairs fertility. On restricted diets, the development of tumours was retarded in rats and mice (Tannenbaum) while the incidence of pneumonia and the frequency of chronic nephrosis were considerably decreased (Saxton). While the effect of restricted diets on tumours and pneumonia was absent in some experiments on rats, the difference in the nephrosis incidence was always present. The incidence of calcification of the heart and kidneys was also higher on an unrestricted food intake: for example, in two experiments on rats kept on this diet it occurred in 55 and 17 per cent respectively, while on the restricted food intake the respective figures were 36 and 4 per cent (Barnes). The statistical analysis of a very large number of the data of Insurance companies (Dublin and Marks; Armstrong *et al.;* Tannenbaum) shows that at every age period the mortality of overweight men and women is considerably greater than that of persons with a normal bodyweight. For example, amongst overweight men, between the ages 20–64 years, the mortality was 50 per cent greater than that of men with normal weight. In particular, mortality from degenerative diseases of the cardiovascular system and kidneys, and from diabetes was much higher, being 149, 191, and 383 per cent respectively in overweight men, against 100 per cent in normal

weight men (Dublin). Tannenbaum emphasises an apparently greater frequency of cancer in overweight persons, as suggested by statistical investigations. For example, in persons with normal weight the mortality from cancer was 111 per 100,000; in persons of 15 to 25 per cent overweight it was from 138 to 143. Dublin and Marks, however, in their later investigation observed that the mortality from cancer in the overweights and normals was about the same.

These authors found that the most encouraging feature was the fact that therapeutical weight reduction improves the health outlook and life span for overweight individuals. For example, amongst the overweight men who reduced their weight by medical treatment, the mortality was 113 per cent of the standard as compared with a mortality rate of 142 per cent for all moderately overweight persons.

Henschen reports a striking example of the effect of food restriction on the mortality from cardio-vascular diseases in the whole nation. Thus, during the last war in Sweden and Finland the caloric food restrictions were rigorous, but there were enough vitamins. This proved to be very favourable, because in general the national state of health was never so good in Sweden as during the war; in particular the mortality from arteriosclerosis and myocarditis chronic decreased to a minimum. After the war, with the removal of food restrictions, the mortality from these diseases increased rapidly and finally even rose to a higher level than before the war.

Summary. Both experiments on animals and observations on man agree in that overfeeding causes premature ageing and death.

A decreased incidence of tumours was found in nearly all experiments on animals on restricted diets, while only in some of the observations made on humans the cancer mortality appeared to be increased in the overweights.

Harmful Effects of High-Protein Diets

Since toxic properties were found in the metabolic products of proteins, the effects of high-protein diets are of especial interest for the problem of autointoxication and gerontology. It must be remembered, however, that proteins constitute a vitally

necessary part of the diet. Therefore it must be expected that the mechanisms for the neutralisation of the possible damaging properties of proteins and their metabolic products should be highly developed in the animal organism. The excretory organs (*e.g.*, the kidneys), metabolism organs, in particular the liver, and some endocrine glands which appear to possess a detoxifying function, should play an important role in these mechanisms.

The efficiency of these neutralising or defense mechanisms is very high. Thus, Folin *et al.*, found that a protein-rich diet, given for 4 or 5 days, did not increase, or increased only a little the non-protein nitrogen in the blood of their patients with a high blood pressure. When, however, a low protein diet was given the non-protein nitrogen dropped from 22–28 mg. per 100 c. c. of the blood to 11–14 mg. Even when kidney lesions, as in nephritis, handicap the excretion of metabolic products, the non-protein nitrogen in the blood might remain in some cases on a normal level (Foster). Only when renal lesions become pronounced, does this nitrogen rise to 50–100 mg. per 100 c.c. of the blood (Boyd). A considerable accumulation of nitrogenous metabolites occurs not only in the blood, but in the tissues as well.

Probably a more or less perfect development of the neutralising mechanisms might partly explain the different response to high protein diets of different individuals, or, in the case of animals, of different strains or species; and, accordingly, some of the contradictory results obtained in different laboratories might be explained in this way.

Thus, in some experiments on rats, high protein feeding produced only a hypertrophy of the kidneys, but no nephrotic or nephritic changes (Addis, Miller). In other experiments, however, renal lesions were pronounced, in some cases amounting to chronic nephritis (Newburgh); and high blood pressure (Handler) or increase of the "artificial" renal hypertension was obtained. The latter effect, however, was not produced in dogs (Guerrant). In rabbits arteriosclerosis was found on protein-rich diets (Anderson).

The damaging effects of these diets was more pronounced when the animals were fed with proteins which normally were not consumed in their natural food, as for example in the case of a meat diet fed to rabbits (Newburgh) or fowl. Even in the case of omnivorous animals, *e.g.*, rat, the type of food protein

(meat, liver, casein or egg albumen) was found to play an important role in producing more or less pronounced lesions (Ingle).

A prolonged duration of feeding with protein-rich diets was observed to be important by nearly all experimentators on the subject.

The age factor was also playing a role; older rats were found to be more susceptible to the injurious effects of a high-protein diet than young rats (Smith and Moise). Saxton and Kymball recorded nephrosis in 50 per cent of old rats when kept on a high casein unrestricted diet, and only 12.5 per cent on a low casein unrestricted diet. No nephrosis was found on a low casein restricted diet.

Some experiments were devised with the aim to break or decrease the mechanisms neutralising the possible damaging effects of abundant protein ingestion. Thus, unilateral nephrectomy was found to be effective in producing or increasing nephritic or arterio-sclerotic lesions.

The protective function of the adrenals was indicated by the experiments of Benua and Howard: the growth of normal unoperated young mice was not retarded by the high-protein diet, but it was decreased by 31 per cent in unilaterally adrenalectomised animals. A normal diet did not retard the growth of the similarly operated control mice. Although Ingle *et al.*, did not find a difference in the survival period of bilaterally adrenalectomised rats kept on a normal diet and those fed on a protein-rich diet, their animals were on the experimental diets for 7 to 28 days only.

Excessive doses of corticotrophic extract of the anterior hypophysis can produce hypertensive disease in rats (hypertension, nephrosclerosis, and adrenal enlargement). This disease was markedly increased on high-protein diets (Shoch). Henriques *et al.*, using another hypophysial preparation, obtained the hypertensive disease only in those rats to which simultaneously a high-protein diet was given.

Some vitamins also appear to have a certain relation to the effects of proteins in the diet, as was observed in the case of nicotinic acid and riboflavine (Sarett), pyridoxine (Miller) and yeast extract. Besides pathological changes in the kidneys and vascural system, atrophic changes in the testes of cocks (Pezard)

and rats (Paul) and sterility of eggs in fowl (Houssay) were produced by high protein diets. Meat feeding was used in these experiments on birds and rats, and therefore a deficiency in some vitamins might also be responsible for the changes obtained.

Squier and Newburgh recorded an irritating effect of a high protein diet on the kidneys of men. This was manifested in normal men by the appearance of red blood cells in the urine; in the patients with high blood pressure the same effect occurred, and also albuminuria or an increase of pre-existing albuminuria and oedema were found.

Summary. The results obtained indicate the existence of efficient defense (neutralising) mechanisms in the organism against the injurious effects of high-protein diets. So far these mechanisms have been studied insufficiently, and not all of them are known. Their development appears to vary in different individuals, strains or species. Apparently, when these mechanisms are insufficiently developed, or are overcome or weakened by various factors, the injurious effects of protein-rich diets are manifested in various pathological changes, chiefly in the kidneys and cardio-vascular system. The duration of feeding, the type of proteins given and the age of animals also play a definite role in producing these harmful effects.

Toxic Effects of Various Metabolites

Amino acids administered simultaneously in groups. These acids, just like proteins, are normal constituents of animal organs and tissues, from which they have been isolated by several investigators. Therefore the examination of their toxicity meets with the same difficulties which have been discussed in the introduction to the previous chapter on high-protein diets.

In addition, as in the case of several other compounds in the organism, some metabolites produce physiological or toxic effects, sometimes a directly opposite action, depending on the size of the dose, as this will be shown in the following.

Excretion of amino acids in the urine occurs normally. For example, the following daily amounts (mg.) were found by Haney and Horwith in human urine: arginine 4.1; histidine 38.0; isoleucine 0.9; leucine 1.0; lysine 5.0; methionine 3.0; phenylalamine 4.7; threonin 4.3; tryptophane 11.3; valine 2.4.

In rats and mice kept on a diet with 10 per cent casein the excretion of amino acids, calculated as the percentage of the ingested amino acids, varies according to Sauberlich and Baumann, from 2.46 for cystine to 0.32 for aspartic acid. The daily excretion of other amino acids varies between the two above-mentioned figures. On diets rich in proteins (*e.g.*, 50 per cent casein) their excretion becomes three to four times greater.

A crude mixture of these acids, prepared as a digest by the acid hydrolysis of casein, was injected intravenously into men and dogs (Elman), and subcutaneously into rats without showing any toxic effects. Bassett *et al.*, injected a mixture of eleven essential pure amino acids to a patient, while Madden *et al.*, administered a similar mixture orally or intravenously to dogs. No toxic effects were observed. In all these experiments, however, it was emphasized that only small amounts of these acids, injected slowly, can be well tolerated.

Henriques *et al.*, as was shown previously, produced hypertensive disease in rats by the administration of a hypophyseal extract only in those animals which were fed on a high protein diet. These authors obtained the same results in rats, in the diet of which the proteins were replaced by 37 per cent of a mixture of twelve essential pure amino acids.

Administration of single amino acids, their derivatives and some other metabolites. Alanine was not found to be definitely toxic by Newburgh and Marsh. Its vasodilating effect was found in experiments on various isolated organs: the kidneys of rabbits (Lussana) and dogs, the spleen, thyroid, and legs of dogs (Brouha).

Arginin and Aspartic acid, intravenously injected in large doses 0.5–2 g. per kilo bodyweight, produced mild degenerative changes in the kidneys of rabbits and dogs.

Cholic acid and glycocoll are derivatives of the bile glycocholic acid. When injected into rats subcutaneously, intraperitoneally or intramuscularly in doses 0.3–15 mg. per 100 g. of bodyweight, MacKay found that they produce depressing and narcotic effects. Glycocoll has a vasodilating effect on various isolated organs of the dog (Brouha).

Choline and acetylcholine, derivatives of lecithine, are vitamin-like substances. Acetylcholine is the ester. Small amounts of these substances are normally present in the blood (*e.g.*, 2–20

mg. of choline per 1000 c.c. of serum). Deficiency in choline produces a pathological fat deposition, cirrhosis, and the development of tumors in the liver (Schaeffer) and hemorrhagic degeneration in the kidneys (Griffith).

Although the deficiency effects were found to be severe, excessive doses of choline and acetylcholine, according to some investigators, also produce pathological changes: in the case of choline (Davis)—anaemia and various lesions in the brain multiple hemorrhages, nervo-vascular gliosis, glial scars and nodules); in the case of acetylcholine (Hall)—degenerative changes in the myocardium, hyaline and fibrous degenerations and thrombosis of the coronary arteries, and the development of tumours. Goldblatt *et al.*, did not observe the above-mentioned heart and coronary lesions in their dogs injected with acetylcholine for 144 days. Hall *et al.*, however, emphasised that they recorded the above-mentioned changes only in older dogs; in young animals the lesions were slight or absent.

It has also to be taken into consideration that different effects were produced by different doses of acetlycholine as was shown by Gaddum and Holtz: small doses dilated, large doses constricted the blood vessels in the lungs.

Some toxic effects were observed in man: headaches, giddiness, weakness, dyspnoe, tachycardia, secretion of tears, and saliva, and muscular tremors (Spatz). Magnus, however, emphasized that commercial preparations of choline might contain toxic impurities.

Creatine and creatinine are normally present in the blood, urine, skeletal and cardiac muscles, and in various organs. In 100 cc. of blood their content, when estimated together, varied from 1 to 1.5 mg. in all children investigated; larger figures, namely, 1.5 to 2.5 mg. were found by Feigl in 26 per cent of adults, and in 49 to 54 per cent of old persons.

When the content of creatine alone was estimated, the effect of the age factor was still more obvious (Fonteyne and Ingelbrecht): in women—2.97 mg. in 100 c.c of blood of young persons, 3.53 mg. in adults, 3.53 in senescene, 3.82 mg. in senility; in men—2.96 mg. in young, 3.04 mg. in adults and 3.67 mg. in senescene.

The feeding of adequate diets, supplemented by creatine, increased its content in the liver of rats from 0.033 up to 0.091

per cent (Chaustin). No effect on the growth of these animals, nor any apparent toxic effects were noted in these creatine fed mice.

Fonteyne and Ingelbrecht found that the blood content of creatinine was increased in renal and some other diseases. When its amount rises to 3.4 mg. or more per 100 c.c. of blood, the prognosis in kidney diseases is very grave.

Cystine and cysteic acid are sulphur-containing amino acids. Cystine is a necessary constituent of the diet, and is normally excreted in the urine in small amounts. Pathologically it may be excreted in large quantities in the cases of systinuria. In Lignac's disease of children (Lignac) it is deposited in considerable quantities in various organs. A hereditary factor plays a caustive role both in cystinuria, which is a disease of adults, and in Lignac's disease of children. Both diseases might occur within the same family.

Excessive amounts of cystine are toxic. Thus, while 0.05 per cent in the food of rats is necessary for their normal growth, ten times this amount becomes harmful. Five per cent of cystine in the diet kills rats in several weeks, while ten per cent does it in a few days according to Curtis and Lillic. Histological changes were found in the liver (fatty infiltrations, hemorrhages, necrotic nests and, in prolonged experiments, cirrhosis), and in the kidneys (parenchymatous degeneration and necrotic nests). According to Earle and Victor, however, renal lesions were rare in their rats. In rabbits (Lewis) and dogs they were pronounced and regular. Young rats and mice are more susceptible than older animals (Lignac). Cysteic acid produces similar changes in the liver according to Earle. Sullivan *et al.*, explained the non-toxic results obtained in the experiments of some investigators by neutralizing effects of a comparatively large amount of yeast in the diet of the rats. Cox and Hudson confirmed the neutralising effect of yeast on the toxic properties (nephrosis) of cystine. Increased amounts of cystine in the diet raise the non-protein nitrogen in the blood.

Glutamic and glutaric acids are derivatives of glutamine. Ten per cent of glutamic acid added to rat diets was found non-toxic (Kutscher) or slightly toxic (Sullivan). The presence of this acid in a mixture of amino acids, intravenously injected into dogs, produced vomiting in some cases (Madden). Sodium glu-

tarate was found by Rose to be nephrotoxic when subcutaneously injected in large doses (1–2 g.) into rabbits.

Glycocoll (glycine) produces slight depressing and narcotic effects on rats injected subcutaneously, intraperitoneally or intramuscularly (McKay), but apparently was not toxic in the experiments on rabbits and dogs (Newburgh). It produced, however, a vaso-dilating effect in various isolated organs of dog (Brouha).

Guanidine. The average content of guanidine-like substances in men is 0.25 mg., and in women 0.23 mg. per 100 c.c. of blood (Barnes); in rabbits 0.56 mg. (Andes). Besides guanidine, methyl- and dimethyl-guanidine were also isolated from human urine (Addis). In various diseases the guanidine concentration usually follows the degree of urea and creatinine concentrations (Andes).

In parathyroidectomy some authors found normal values for guanidine in the urine or blood (Andes, Greenwold, Raida) while others observed increased amounts (Burns), but their method of guanidine estimation was criticised.

The symptoms in parathyroidectomised animals, and those injected with guanidine are similar. This was responsible for the theory explaining the parathyroidectomy effects by guanidine accumulation in the blood and tissues (Koch).

Toxic doses of guanidine produce the following symptoms in injected animals (Klinger, Koch): fatigue, muscular tremors and twitchings developing, in severe cases, into clonic cramp, prolonged rise of the blood pressure, irregular heart beats, slow rates of respiration, prostration and death. Histologically necrotic centres in some lobules, with fatty degeneration at their periphery, were found in the liver; in the kidneys and adrenals—congestion and necrotic nests; in the spleen—great congestion and the accumulation of blood pigment (Larson).

Histidine and Histamine. The *histidine* content was found to be normal in old age (Ackerman). When injected intravenously into rabbits and dogs in doses of 0.1–0.3 g. per kilo bodyweight, it produced serious kidney lesions: congestion, degenerative changes in the tubular epithelium, pycnosis and sometimes the disappearance of nuclei, hemorrhagic and necrotic nests, casts and calcification in some areas (Newburgh).

Histamine is a derivative of histidine. It is normally present in the blood and tissues, *e.g.*, 2.7–5 mg. were found in 100 c.c. of human blood (Barsum). General symptoms produced by traumatic shock, anaphylaxis and burns were explained by the excessive liberation of histamine from the injured tissues.

The chronic administration of histamine to rabbits, guinea-pigs and rats produced chiefly cardio-vascular lesions (Farmer), similar to those in dogs which survived severe histamine shocks (Hueper). In the aorta and smaller arteries degenerative oedematous, cystic and calcifying changes, and inflammatory cellular infiltrations were observed. In guinea pigs a thickening of the muscular layer of the arteries was also found. All these changes might result, in some cases, in a complete occulusion of blood vessels. Oedema, cellular infiltrations, fibrous tissue development, and small nests of calcifications were observed in the myocardium and endocardium. Coronary arteries might be involved. Subepicardial and subendocardial hemorrhages were also recorded. These vascular changes may result in gastric ulcers, nests of superficial intestinal necrosis, hepatic pericentral nests of necrosis with hemorrhages and fibrous growth, and fibroblastic proliferations in the spleen (Furth).

In human beings, with comparatively small doses used, the symptoms of histamine poisoning include headache, congestion in the head, nervous depression, increased secretion of glands, dyspnoea, cardiac arythmia, fall of blood pressure, nausea and finally collapse (Cushny).

Indol and scatol are derivatives of tryptophan and usually originate from protein putrefaction in the intestines. Both are toxic and, in chronic experiments, the *total* lethal dose for rabbits varied from 0.15–0.22 g. (Herter) to 1.5–2.0 g. (Rovighi), with a daily subcutaneous injection of 10 mg. The following symptoms of poisoning were observed (Herter): decrease of temperature, somnolence, prostration, paresis, cardiac weakness, loss in bodyweight, sometimes muscular spasms or cramp and, finally, death. Histologically congestion, round cell infiltration, degenerative changes, and pigment deposition in the cells were found in the liver, and congestion in the kidneys. A proper histological examination of all organs was not performed.

Lysin was found to be nephrotoxic to rabbits in the dose of 0.15 g. per kilo bodyweight, and to dogs in the dose of 0.6 g. per kilo (Newburgh).

Pyruvic acid, like lactic acid, is a normal intermediate product of carbohydrate metabolism, and is alson contained, as a constituent, in some amino acids (alanine, serine and tryptophan). Pyruvic acid is normally present in the blood in a concentration of less than 3 mg. per 100 c.c. Its contents is increased four or five times in the blood of Vitamin B deficient animals and man (Mitchell). According to Dobrovolskaia, subcutaneous injections of 0.2 c.c. of a 10 per cent solution sodium pyruvate into mice produce cramps and convulsions followed by death. Histologically parenchymatous degenerative changes in the hepatic cells and perivascular oedema in the brain are found.

Serine. Administration by stomach tube of 100 mg. serine to rats for about 14 days produces dilation and degenerative changes of the tubules, nests of necrosis and calcification in the kidneys; fatty infiltration and hemorrhagic transudations in the liver, and myocardial lesions in the heart (Morehead).

Taurine. Although Salkowsky found taurine to be toxic, Schmidt *et al.*, and Earle *et al.*, could not confirm this even with large doses given to humans, rabbits or rats.

Tryptophan. The concentration of this essential amino acid was found by Ackerman to be about the same in the plasma of young and old men. Hicks observed that 0.5 per cent of tryptophan, added to a diet deficient in this amino acid, caused rats to resume normal growth, but 2 or 3 per cent started to produce some toxic effects. The general toxicity and nephrotoxic effects of tryptophan were recorded in dogs, rabbits (Newburgh), and rats. In these experiments large doses were used: 20 per cent added to the diet, or 0.65–0.85 g. per kilo bodyweight by intravenous injection. Chang, however, found it non-toxic for rats subcutaneously injected with 10 to 20 mg. daily during 22–111 days.

Tyrosine. Medes described a metabolism disease "tyrosinosis," in which the oxidation of tyrosine in the organism stops completely at the stage of hydroxyphenylpyruvic acid. As the result, this acid and tyrosine itself are excreted in large quantities in the urine. Hueper and Martin, summarizing the clinical

literature on the subject, emphasized that tyrosinaemia was recorded in the diseases of the biliary system, and that pancreatitis and diabetes occur often as the so-called "second diseases" in these conditions. As a precursor of tyramine, tyrosine has been related to the origin of "pale hypertension," while, as the chief component of melanin, it has been suspected as a factor responsible for the incidence of primary cancer of the liver among the coloured races.

Experimentally, toxic effects were produced by this essential amino acid only when it was administered in amounts which exceed by a wide margin the physiological needs. When 5–10 per cent was added to the diet, rats died in most cases within a week (Sullivan). Lillie investigated these rats histologically and found degenerative changes in the kidneys, in some animals hepatic fatty infiltrations, exudative blepharitis and a central degeneration of the eye-lenses.

Hueper and Martin's rats, kept on a diet to which 10 per cent tyrosine was added, died within 3 to 5 weeks, showing purulent keratitis, and swelling and redness of their feet and legs. The following changes were observed histologically: in the kidneys—degenerative changes with nests of necrosis and cystic distension of the tubules, and cellular infiltrations; degenerative, necrotic and fibrous changes in the pancreas; atrophic changes in the testicular epithelium with the formation of giant cells; in the arterioles of the brain, heart, lungs, and kidneys—swelling and hyalinisation of the media, and sometimes proliferation of the intima; in the brain, besides vascular changes, focal hemorrhages and necroses, and glia cell accumulation. Doses of 0.23 g. and 1 g. per kilo body weight, injected intravenously into rabbits and dogs respectively were also nephrotoxic (Newburgh).

Valine. According to Ackerman, valine content is not increased in old age. Its vasodilating properties were recorded in experiments on isolated organs (Brouha).

Toxicity of purines. Purines are derivatives of nucleic acid. Like amino acids, they are naturally present in the blood and organs. Some of them, namely, adenine, adenosine, adenosine triphosphate and adenylic acid, in small doses, possess important physiological properties, the most essential being their vasodilating effect. Drury, in his comprehensive review on the sub-

ject, summarises the physiological activity of purines as follows: they slow the heartbeat, lower the blood pressure, inhibit intestinal muscle, stimulate the uterus, and produce leucocytosis.

The adenine content in human blood is 10.5–30 mg. per 100 c.c., in rat 6.5–9.5 mg., in dog 7–12 mg., in pigeon 64–85 mg. (Buell). High levels were observed in cases of essential hypertension by Zander. This suggests the probability that adenine is one of the constituents of the natural mechanism neutralising the pathological effects of hypertension.

In larger amounts, however, purines were found to be toxic, producing chiefly a severe heart block and probably some other pathological changes so far not investigated. How close the toxic dose is from the physiological one is shown by Gillespie in experiments on perfused rabbit hearts: 0.1 mg. of adenosine triphosphate produced a heart block, while 0.01 mg. was necessary for the physiological improvement of the heart beat and the dilation of coronary vessels. Moreover, the opposite effects on some organs might be obtained by various doses of adenosine: *e.g.*, small doses dilate, while large doses constrict the blood vessels of the lungs, according to Gaddum and Holtz.

Adenosine was found by Lindner and Rigler to be low in patients with angina pectoris.

Toxicity of abnormal metabolites. This problem is practically uninvestigated, although it is very important. In connection with it, the experiments of Fasciola *et al.*, can be mentioned. They found that ischaemic kidneys produce some abnormal vaso-constricting and blood pressure raising substance, so far unidentified.

There are many metabolites, normal and abnormal, several of which are still not investigated, or are unidentified or even unknown. Comparatively few of the known normal metabolites have been studied, but in most cases insufficiently, especially in relation to their toxic properties.

These properties are difficult for investigation chiefly due to the naturally existing neutralising and defense mechanisms, varying effects of different doses, and also because of some other causes discussed. Besides these difficulties, a possibility of toxic impurities in the preparation of metabolites has also to be taken into consideration. All these factors might explain the con-

tradictory results obtained by different investigators in the case of certain metabolites.

However, some other metabolites (in particular cystine, cysteic acid, guanidine, histamine, indol, scatol, typrosin, adenine, adenosine, its triphosphate compound and adenylic acid), when used in increased non-physiologic amounts and especially for prolonged periods, were found to be toxic in varying degrees by all the investigators who studied them.

Various metabolites produced more or less pronounced changes in the various organs and tissues investigated, chiefly in the kidneys, liver and cardio-vascular system. In particular various forms of nephrosis, nephritis, liver cirrhosis, arteriosclerosis including that of the coronary vessels, hypertensive disease, gliosis of the nervous system and some special syndromes (*e.g.*, cystinuria, Lignac's disease, tryosinosis) were observed.

The effect of age on the levels of metabolites in the blood and tissues was hardly investigated, in spite of the primary importance of this problem. So far only the contents of creatine, creatinine, histidine, tryptophan and valine in the blood of old persons have been assayed. An increased accumulation of creatine in old age was found.

Toxic Products of Gastro-Intestinal Putrefaction

Another source of toxic substances in the organism is created by the putrefaction of proteins and their derivatives in the gastro-intestinal canal, chiefly in the colon. Metchnikoff, in his numerous and well known studies, emphasizes that the products of putrefaction poisons are absorbed from the intestinal contents during the whole of life, slowly but steadily producing their toxic effects. These effects are intensified during the periods of increased putrefaction; or when its poisons accumulate in the intestines and, after their absorption, in the blood and tissues, which usually happens during periods of more or less pronounced constipation.

At the same time Metchnikoff's attention was drawn to the observations made in some Southern European countries, in which a long span of life appears to occur comparatively often.

In these countries the consumption of yoghourt, sour milk fermented by special lactic bacteria, in particular by Bact. Bulgaricum, is wide-spread. The lactic bacteria, including those of yoghourt, inhibit the putrefaction both *in vitro*, and in the intestines. On these observations Metchnikoff built his theory. He writes: "If it is true that our precocious and unhappy old age is due to poisoning of the tissues (the greater part of the poison coming from the large intestine inhabited by numberless microbes), it is clear that agents which arrest intestinal putrefaction (*e.g.*, lactic bacteria, V. K.) must at the same time postpone and ameliorate old age. This theoretical view is confirmed by the collection of facts regarding races which live chiefly on soured milk, and amongst which great ages are common." Metchnikoff urges that his theory needs to be tested by numerous systematic scientific investigations, in particular in the infirmaries for old people.

Metchnikoff's ideas and hopes were exaggerated. Orla-Jensen *et al.*, however, are right when they conclude that this theory, like all great ideas, has not been proposed in vain (p. 8). The theory that the intestinal putrefaction might be one of the many secondary causes of ageing, was already supported by some investigations. Thus, Orla-Jensen *et al.*, counted various types of bacteria in the feces of young, senescent and senile men. The degree of putrefaction was assayed by the ratio of Bact. bifidum (which is the predominant natural lactic bacterium in the intestinal flora of milk fed children) to the putrefying bacteria which invade the intestinal contents with ageing. The following abbreviated table 1 shows that in old men the putrefaction is greatly increased in the intestines. This fact, the authors conclude, confirms Metchnikoff's idea.

TABLE 1

Relation of Intestinal Putrefaction with Age

Groups of investigated persons	Per-cent of persons having more than 1000 millions per gram of feces, of:—		Degree of putrefaction as calculated both from putrefactive bacteria and their spores
	Bacterium bifidum	Putrefying bacteria	
Young	100	0	0
Senescent	44	9	40
Senile	9	48	55

Unfortunately so far the poisonous substances produced by putrefaction in the intestines have been investigated only in a few cases and insufficiently. In addition to scatol and indol, described in the previous chapter, the toxins produced by Bact. Welchii (Bac. perfringens) have been investigated by Evans, by Korenchevsky and by Metchnikoff. This microbe and Bac. putrificus are mainly responsible for the putrefaction in the intestines. The toxin of Bac. Welchii produces severe symptoms of depression, clonic convulsions, intermittent with paresis, locally gangrene and, with sufficiently large doses, death in a few hours. The toxin of Bac. putrificus is weak, as shown by Korenchevsky.

The effects of sour milk bacteria on the processes of ageing have not yet been investigated with the necessary research control and technique. There is, however, an interesting clinical investigation by Rettger and associates relating to the problem. The authors have shown that by the administration of Lactobacillus acidophilus (which is probably identical with Bact. bifidum) it is possible to change favourably the putrefactive intestinal flora in man; and that such treatment results in the improvement or cure of about 75–87 per cent of patients suffering from constipation alone, or simultaneously with some biliary symptoms. They state also that cases of mucous or ulcerative colitis are improved. Many patients were in great distress, pain and in a state of exhaustion, and in some cases were emaciated. Rettger's results have been confirmed in Germany and Denmark by Orla-Jensen and associates.

Apparently it is possible to reduce to some extent the putrefactive bacteria in the intestines not only by lactic bacteria, but also by the consumption of large quantities of lactic sugar. This is explained by the fact that the lactic sugar, in contrast to other sugars, is not absorbed in the small intestines but passes as far as the colon. In the latter it is easily fermented by those lactic bacteria which still naturally remain in the large intestines. In this way, lactic sugar stimulates the rapid development of lactic flora, and finally causes its predominance over putrefactive bacteria.

Autointoxication with Metabolic Waste Products in Isolated Tissue Cultures

The well-known experiments on the subject by Carrel and his associates appear to be of great importance in support of the theory of the causative role of autointoxications in premature ageing and death. These experiments were extended by several other workers, and thir significance was considered by Medawar and Korenchevsky.

All these experiments have shown that in the serum of old individuals there is an increased amount of substances which inhibit the multiplication and growth of cells. "Old" human serum not only depresses the growth of human embryonic tissues, but also produces their degeneration in 52 per cent on the seventh day of their growth, and in 100 per cent on 11–13th day as observed by Weitzmann.

However, in cells isolated from the organism and kept alive *in vitro* as a tissue culture, senescence and death could be greatly postponed ("indefinitely" as suggested by Carrel) if, by frequent changes of the medium, the metabolic waste products were not allowed to accumulate. In such conditions the "old" cells (*i.e.*, those from older animals) have just as high a capacity for growth as young ones. The old cells merely take a longer time than do the young ones in starting their growth. Pearl, similarly to Carrel, concludes that "senescence appears not to be a primary attribute to the physiological economy of cells as such," and "that the individual cells and tissues of the body, in and by themselves, are potentially immortal."

More experiments, however, are needed in order to link Carrel's results with the autointoxication problem of old age in an undisputable way, so that the results obtained cannot be explained otherwise. For example, Weitzmann observed that the addition of the embryonic extract to the tissue cultures, which grew in "old" serum, improved to a certain extent their growth. This improvement indicates that not only the accumulation of waste products, but a deficiency in some nutritive substances in the blood of old individuals might also be partly responsible for depressive effects of "old" serum.

Effects of "Blood-Washing" by Isotonic or Slightly Hypertonic Solutions

If in old age the metabolites accumulate in excessive and harmful amounts in the blood and tissues, it is reasonable to expect that these abnormal amounts might be washed out from the organism, more or less completely, by introducing large quantities of suitable watery solutions into the blood.

Such a treatment has so far been used in cases of thromboangiitis obliterans and arteriosclerosis of the periphery blood-vessels. It is remarkable that the authors, who used this procedure, concentrated their attention on the local changes in these diseases. They have paid little or no attention to the effects of this treatment on the general processes of ageing, nor have they considered the possibility of autointoxication with metabolites in these cases. Accordingly, the results obtained by this treatment were explained not by the removal of the toxic metabolites from the organism, but otherwise, for example, by a decrease of the blood viscosity. Samuels, in his exhaustive book on the subject, considers the latter explanation inadequate and states that the true explanation is still unknown.

Two methods were used in this treatment, namely, the "artificial kidney," as suggested by Merrill and associates, and the parenteral or intravenous injections of large amounts (100 to 500 c.c.) of physiological or Ringer's solutions (Koga), or 2 per cent sodium citrate solution (Bernheim), or hypertonic 2–5 per cent sodium chloride solutions as recommended by Samuels. By both methods considerable amounts of metabolites can be removed from the blood. Thus, in the experiments with the artificial kidney of Merrill *et al.*, from 10 to 12 grams of urea were washed out from the blood per hour, and from 210 to 633 mg. of amino acid nitrogen. At the same time by this treatment the blood viscosity (as measured by Hess' viscosimeter) was decreased from 5.4–8.0 to 4–5.5.

The intravenous injections can be repeated, if necessary, daily for several months, without any apparent hazard for the patients, provided they do not suffer from hypertension, auricular fibrillation, myocardial damage or kidney impairment. These diseases are contraindications against the procedure.

The following favourable results were obtained in cases of thrombo-angiitis obliterans and arteriosclerosis of the peripheral bloodvessels: locally—disappearance of cyanosis, increased warmth of the extremities, reopening of obliterated vessels, healing of ulcers and gangrene, improvement in growth and condition of deformed nails, ability to walk longer distances without cramp or pain; in the general condition—gain in weight, improved nervous condition, better sleep and appetite, and healthy rosy colour of the face.

Bernheim and London and Silbert claimed an improvement in about 80–83 per cent of the treated patients. The improvement may be lasting, or only temporary, disappearing with the discontinuation of the treatment.

Very few experiments on animals have been reported. Carrel performed a striking but drastic experiment on one dog only, and this probably was the reason why the experiment was published not by him, but by Du Nouy, one of his coworkers. At the Rockefeller Institute there was a dog nearly 18 years old. The animal never stirred from its corner and could hardly get up to eat. It slept all day, its hair was coming out, its eyes were dim, and the eyelids were stuck together. Nearly two-thirds of its blood was removed, the red cells centerifuged, washed in Ringer solution, then mixed with fresh Ringer solution sufficient to re-establish the initial volume of the blood and reinjected into the dog. After recuperation the operation was repeated. After the second operation the dog not only lived but, when completely recovered, was quite different: it ran and barked, became lively and active—in particular, sexually active—its lids cleared and its fur started to grow again. The relief was only temporary. Unfortunately nobody has repeated this experiment, which greatly undermines its otherwise great significance.

Saxton *et al.*, in McCay's laboratory, found that increased levels of fluid ingestion (coffee, milk, sucrose solution or water) did not appreciably alter the life span of treated rats. It may be possible to introduce intravenously more than 500 c.c. daily into patients, since Goldblatt *et al.*, injected this amount daily for about five months into adult dogs without any harmful effects.

The results obtained by "blood-washing" with isotonic or slightly hypertonic solutions appear to provide another indirect support to the theory that autointoxication by metabolites might

be one of the causes of premature ageing. By "blood-washing" procedures it is possible to remove large amounts of metabolites from the blood and tissues.

The special importance of these results is in the fact that they suggest one of the practicable methods of geriatric treatment which might be used both for preventive and curative purposes. The solutions, however, used for "blood-washing" (*e.g.*, physiological saline or Ringer's solutions) were not the best possible for the purpose; or, as in the case of hypertonic solutions, they possibly were harmful in a certain way, since old persons do not tolerate well high sodium chloride intake.

A special "washing" solution has to be devised with an electrolyte content more close to that of the human blood, and with the addition of some blood serum protein so that the "washing" procedure would be tolerated by the blood and other tissues in the best possible way.

At present, this treatment is handicapped by the contraindications against its use in those cases of old age which are complicated by usual and frequent degenerative diseases of old age. Special experimental and clinical research on the technique of "blood-washing" and its effects on all main processes of ageing is urgently needed.

General Summary

In the present study those available data were collected and considered which suggest that autointoxications by metabolic products and products of intestinal putrefaction might be some of the causes of premature ageing and death.

Overfeeding increases the level of metabolites in the blood and tissues of the organism, at the same time increasing the incidence and early appearance of degenerative diseases of old age, and causing premature death. Contradictory results are observed in experiments on the effects of high protein diets. However, their harmful effects were recorded by most investigators when the diet was given for a prolonged period, the neutralising mechanisms were made deficient, older animals were used, and the type of proteins ingested was considered.

Some of the amino acids, purines, and their derivatives, and some other metabolites were found to be toxic in varying degrees,

when these substances were administered at levels exceeding the physiological limits. Some products of intestinal putrefaction were also found to be toxic.

Toxic metabolites produce varying pathological changes in several organs and tissues, in particular in the kidneys, cardiovascular system and liver. These changes include various forms of nephrosis, nephritis, myocarditis, lesions of the blood vessels, in particular coronary or general arteriosclerosis, liver cirrhosis, gastric ulcers, parathyroidectomy-like features, gliosis of the brain, and even tumours.

As has been observed in the case of many other compounds (*e.g.*, hormones, cytotoxic sera, arsenic, etc.), small doses of certain metabolites produce physiological effects, while larger doses may have abnormal, in particular toxic effects.

In the blood of old men the content of only five metabolites has so far been investigated, of which the creatine level was found to be considerably increased when compared with that in young persons.

Frequent changes of the nutritional medium in isolated tissue cultures prevent their ageing, probably due chiefly to the removal of cellular waste products, while "old" cells (*i.e.*, taken from old animals) apparently were made, by this procedure, similar to the young ones.

Intravenous injections of physiological saline, Ringer's slightly hypertonic, and sodium citrate solutions were used for the treatment of older persons suffering from thrombo-angiitis obliterans and arteriosclerosis of the peripheral blood vessels. In spite of deficiencies in the technique used, particularly in the composition of "blood-washing" solutions, in most cases not only the improvement or cure of the local lesions was obtained, but the general condition of the patients also became better. Although these improvements were explained by the lowering of the blood viscosity, they appeared more likely to be the result of "washing out" the metabolites which were accumulated in excessive quantities in the blood and tissues, and which were removed in large amounts by these "blood washing" procedures.

In milk-fed children the intestinal bacterial flora consists nearly exclusively of lactic bacteria (in particular Bact. bifidum). With ageing it is invaded by putrefactive bacteria and some other microbes.

In the intestines of old persons the putrefactive bacterial flora and processes of putrefaction were found to be considerably increased. The administration of lactic bacteria with the food (*e.g.*, in the form of milk fermented by these bacteria) prevented or eliminated, more or less completely, intestinal putrefaction processes by the replacement of the putrefactive bacteria by lactic microbes.

All the above mentioned data were inadequately investigated and are of an indirect nature for definite gerontological conclusions. Nevertheless seven different lines of investigation point in the same direction, namely, the results obtained with overfeeding, high-protein diets, toxicity of some metabolites, "washing out" of metabolites from the blood (and presumably tissues), increased intestinal putrefaction in old age, effects of lactic bacteria on these processes, and experiments with isolated tissue cultures. All these results, when considered together, strongly suggest that autointoxication by metabolites and the intestinal putrefaction products might be one of the many causes of premature ageing and death. Therefore extensive gerontological research of these problems is urgently needed.

REFERENCES

Achelis, W.: Ueber das Vorkommen von Methylguanidin im Harn. *Zschr. f. Physiol. Chem.*, 50: 10–20, 1906–1907.

Ackermann, P., L. Hofstatter, and W. B. Kountz: Concentration of Free Valine, Tryptophane and Histidine in Plasma of Young and Old Individuals. *J. Lab. Clin. Med.*, 34: 234–238, 1949.

Addis, T., E. M. MacKay, and L. L. MacKay: The Effect on the Kidney of the Long Continued Administration of Diets Containing an Excess of Certain Food Elements. *J. Biol. Chem.*, 71: 139–156, 1926–27.

Anderson, H.: Experimental Renal Insufficiency. *Arch. Int. Med.*, 37: 297–312; 313–335, 1926.

Andes, J. E., F. L. Hawk, and M. T. Emerson: Blood Guanidine After Repeated Intoxication With Arsphenamine and Neoarsphenamine. *Arch. Interne. de Pharmac. et Ther.*, 58: 165–174, 1938.

Andes, J. E., C. B. Linegar, and V. C. Myers: Blood Guanidine in Nitrogen Retention and Hypertension. *J. Lab. and Clin. Med.*, 22: 1209–1216, 1937.

Andes, J. E., W. V. Morgantown, and V. C. Myers: Blood Guanidine in Patients With Parathyroid Deficiency and Idiopathic Tetany. *J. Lab. and Clin. Inv.*, 23: 123–126, 1937.

Armstrong, D., L. I. Dublin, G. M. Wheatley, and H. H. Marks: Obesity and Its Relation to Health and Disease. *J. Amer. Med. Asso.*, 147: 1007–1014, 1951.

Barnes, LeRoy L.: The Deposition of Calcium in the Hearts and Kidneys of Rats in Relation to Age, Source of Calcium, Exercise and Diet. *Amer. J. Path.*, 18: 41–47, 1942.

Barsum, G. S., and J. H. Gaddum: The Effect of Cutaneous Burns on the Blood Histamine. *Clin. Science*, 2: 357–361, 1936.

Bassett, S. H., R. R. Woods, F. W. Shull, and S. C. Madden: Parenterally Administered Amino Acids as a Source of Protein in Man. *New Eng. J. of Med.*, 230: 106–108, 1944.

Benua, R. S., and E. Howard: The Effect of Dietry Protein on Adrenal Weights and on Growth after Unilateral Adrenalectomy. *Endocrinology*, 36: 170–177, 1945.

Bernheim, A. R., and I. M. London: Arteriosclerosis and Thrombo-Angiitis Obliterans. *J. Amer. Med. Asso.*, 108: 2102–2109, 1937.

Blatherwick, N. R., and E. M. Medlar: Chronic Nephritis in Rats Fed High Protein Diets. *Arch. Int. Med.*, 59: 572–596, 1937.

Blatherwick, N. R., E. M. Medlar, J. M. Connolly, and P. J. Bradshaw: Nephritis in Unilaterally Nephrectomised White Rats Living Upon High Protein Diet. *J. Biol. Chem.*, 92: Proc. 84–85, 1931.

Boyd, F. D.: Non-protein Nitrogen of the Blood, Especially in Its Relation to Nephritis and Renal Function. *Edinb. Med. J.*, 16: 265–274, 1916.

Brouha, L., et H. Fredericq: Action Vaso-Motrice des Acids Aminés sur Quelques Organs Isolés. *C. R. Soc. Biol.*, 90: 634–636, 1924.

Brunschwig, A., D. E. Clark, and N. Corbin: Postoperative Nitrogen Loss, and Studies on Parenteral Nitrogen Nutrition by Means of Casein Digest. *Ann. Surg.*, 115: 1091–1105, 1942.

Buell, M. V., and M. E. Perkins: Adenine Nucleotide Content of Blood With a Micro-analytical Method for Its Determination. *J. Biol. Chem.*, 76: 95–106, 1928.

Burns, D., and J. S. Sharpe: Tetania Parathyreopriva: Its Nature, Cause and Relation to Idiopathic Tetany. *Quart. J. Exp. Physiol.*, 10: 345–354, 1917.

Carlson, A. J., and F. Hoelzel: Apparent prolongation of the Life Span of Rats by Intermittent Fasting. *J. Nutrition*, 31: 363–375, 1946.

Carlson, A. J., and F. Hoelzel: Growth and Longevity of Rats Fed Omnivorous and Vegetarian Diets. *J. Nutrition*, 34: 81–95, 1947.

Carlson, A. J., and F. Hoelzel: Overnutrition, Its Causes and Consequences. Papers in Honor of R. W. Keeton, 69–78, 1949. C. C. Thomas, Springfield.

Carrel, A., and A. H. Ebeling: Age and Multiplication of Fibroblasts. *J. Exp. Med.*, 34: 599–623, 1921.

Carrel, A., and A. H. Ebeling: Heterogenic Serum Age, and Multiplication of Fibroblasts. *J. Exp. Med.*, 35: 17–38, 1922.

Carrel, A.: *The Problem of the Prolongation of Life*. New York, 1937.

Chang, H. C.: Relation of Tryptophane to Thyroid Activity in the White Rat. *Amer. J. Physiol.*, 73: 275–286, 1925.

Chanutin, A.: A Study of the Effect of Creatine on Growth and Its Distribution in the Tissues of Normal Rats. *J. Biol. Chem.*, 75: 549–557, 1927.

Chanutin, A., and H. H. Beard: A Study on the Effect of Feeding Creatine on Growth and Its Distribution in the Liver and Muscle of Normal Mice. *J. Biol. Chem.* 78: 167–180, 1928.

Christensen, K.: Renal Changes in the Albino Rat on Low Choline and Choline Deficient Diets. *Arch. Path.*, 34: 633–646, 1942.

Cowdry, E. V.: *Problems of Ageing*. 2nd Edition, Williams and Wilkins, Baltimore, 1942.

Cox, G. J., and L. Hudson. The Nephropathogenic Action of Cystine. *J. Nutrition*, 2: 271–276, 1930.

Cox, G. J., P. V. Smith, and C. F. Fishback: The Nephropathogenic Action of Cystine. *J. Biol. Chem.*, 82: 95–103, 1929.

Curtis, A. C., and L. H. Newburgh. The Toxic Action of Cystine on the Liver of the Albino Rat. *Arch. Int. Med.*, 39: 828–832, 1927.

Curtis, A. C., L. H. Newburgh, and F. H. Thoams. The Toxic Action of Cystine on the Kidney. *Arch. Int. Med.*, 39: 817–827, 1927.

Curtis, A. C., L. H. Newburgh, and F. H. Thoams. The Toxic Action of cystine on the kidney. *Arch. Int. Med.*, 39: 817–827, 1927.

Cushny's Pharmacology and Therapeutics, 519–525. Revised by A. Grollman, and D. Slaughter. J. and A. Churchill Ltd., London, 1947.

Davis, J. E.: The Experimental Production of a Hyperchromic Anemia in Dogs Which Is Responsive to Anti-pernicious Anemia Treatment. *Amer. J. Physiol.*, 142: 402–406, 1944.

Davis, J. E.: Evidence That the Hemolytic Anemia by Fat and Choline Is Not Due to Lipotropic Action. Science, 105: 42–44, 1947.

Davis, J. E., and D. E. Fletcher: Nervous System Changes Produced in Dogs by Choline and Carbonyl-Choline. *J. Pharm. and Exp. Ther.*, 88: 246–253, 1946.

Dobrovolskaia-Zavadskaia, N., and V. Momsikoff: Modifications Histologiques du Foie au Cours du Pyruvisme Experimental Chez la Souris. *C. R. Acad. Sc.* (Paris), 223: 1024–1026, 1946.

Drummond, J. C., G. P. Crowden, and E. L. Hill: Nutrition on High Protein Dietaries. *J. Physiol.*, 56: 413–420, 1922.

Drury, A. N.: The Physiological Activity of Nucleic Acid and Its Derivatives. *Physiol. Rev.*, 16: 292–325, 1936.

Dublin, L. I., and H. H. Marks: Overweight Shortens Life. *Statist. Bull. of Metropol. Life Insur. Co.*, 32: 1–4, 1951.

Du Nouy, L.: *Biological Time.* Methuen and Co. Ltd., London, 1936.

Earle, D. P., K. Smull, and J. Victor: Effects of Excess Dietary Cysteic Acid, dl-methionine and Taurine on the Rat Liver. *J. Exp. Med.*, 76: 317–324, 1942.

Earle, D. P., and J. Victor: Cirrhosis of the Liver Caused by Excess Dietary Cystine. *J. Exp. Med.*, 73: 161–171, 1941.

Earle, D. P., and J. Victor: The Effect of Various Diets on the Liver Damage Caused by Excess of Cystine. *J. Exp. Med.*, 75: 179–195, 1942.

Elman, R., and D. O. Weiner: Intravenous Alimentation With Special Reference to Protein (Amino Acids) Metabolism. *J. Amer. Med. Asso.*, 112: 796–802, 1939.

Engeland, R.: Ueber den Nachweiss Organischer Basen in Harn. *Zschr. f. Physiol. Chem.*, 57: 49–64, 1908.

Evans, D. C.: The Protective Properties of the Alpha Antitoxin and Antihyaluroridise Occurring in Cl. Welchii Type A Antitoxin. *J. Path. and Bact.*, 55: 427–434, 1943.

Farmer, L., and G. L. Rohdenburg: Histamine Induced Cardiovascular Lesions. *J. Allergy*, 21: 120–132, 1950.

Fasciolo, J. C., B. A. Houssay, and A. C. Taquini: The Bloodpressure Raising Secretion of the Ischaemic Kidney. *J. Physiol.*, 94: 281–293, 1938.

Feigl, J.: Ueber das Vorkommen von Kreatinin und Kreatin im Blute bei Gesunden und Kranken. III. Weitere Beiträge zur Kenntniss der Norm, insonderheit bezüglich des höheren Lebensalters. *Biochem. Zschr.*, 87: 1–22, 1918.

Folin, D., W. Denis, and M. Seymour: The Non-protein Nitrogenous Constituents of the Blood in Chronic Vascular Nephritis as Influenced by the Level of Protein Metabolism. *Arch. Int. Med.*, 13: 224–234, 1914.

Fonteyne, P., et P. Ingelbrecht: Etude sur la Teneur du Sang en Créatine et en Créatinine Chez l'homme Normal et Chez l'homme Malade. *Ann. de Medicine*, 14: 470–500, 1923.

Foster, N. B.: The Increase of Extract Nitrogen in the Tissues With Chronic Nephritis. *Arch. Int. Med.*, 24: 243–246, 1919.

Fredericq, H., and L. Brouha: Action Vasodilatrice des Acides Aminés sur le Rein Isolé du Chien. *C. R. Soc. Biol.*, 89: 665–667, 1923.

Freudenberg, E.: Cystinosis: Cystine Disease (Lignac's Disease) in Children. *Advances in Pediatrics*, 4: 265–292, 1949.

Fürth, O., and R. Sholl. ueber den Einfluss des Tryptophans und Histidins auf durch Histamin herforgerüfene Magengeschwüre. *Wien. klin. Wchsr.*, 50: 1353–1356, 1937.

Gaddum, J. H., and P. Holtz: The Localization of the Action of Drugs on the Pulmonary Vessels of Dogs and Cats. *J. Physiol.*, 77: 139–158, 1933.

Gillespie, J. H.: The Biological Significance of the Linkages in Adenosine Triphosphoric Acid. *J. Physiol.*, 80: 345–359, 1933.

Goldblatt, H., J. R. Kahn, and H. A. Lewis: Studies on Experimental Hypertension. XVII. Experimental Observations on the Treatment of Hypertension. *J. Amer. Med. Asso.* 119: 1192–1201, 1942.

Greenwald, I.: Are Guanidines Present in the Urines of Parathyroidectomised Dogs. *J. Biol. Chem.* 59: 328–337, 1924.

Griffith, W. H.: The Relation of Choline to the Kidneys. *Biolog. Symposia*, 5: 193–212, 1941.

Guerrant, J. L., J. K. Scott and J. E. Wood: Effect of High-protein Diet and Urea Administration on the Blood-pressure of Normal Dogs and Dogs With Experimental Renal Hypertension. *Amer. Heart J.*, 26: 232–238, 1943.

Hall, G. E., G. H. Ettinger, and F. G. Banting: An Experimental Production of Coronary Thrombosis and Myocardial Failure. *Canad. Med. Asso. J.*, 34: 9–15, 1936.

Hall, G. E., and W. R. Franks: Physiological Carcinogenesis. *J. Biol. Chem.*, 123: XLIX–L, 1938.

Handler, P., and F. Bernheim: Dietary Factors in Experimental Renal Hypertension. I. Protein. *Federat. Proc.*, 7: 289, 1948.

Harvey, C. C., and M. K. Horwith: Excretion of Essential Amino Acids by Men on a Controlled Protein Intake. *J. Biol. Chem.*, 178: 953–962, 1949.

Henriques, O. B., S. B. Henriques, R. Degrandpré, and H. Selye: Influence of Amino Acids on Adrenal Enlargement, Nephrosclerosis and Hypertension by Anterior Pituitary Preparations. *Proc. Soc. Exp. Biol. and Med.*, 69: 591–593, 1948.

Henschen, F.: Pathogenesis of Arteriosclerosis in the Light of Food Restriction in Scandinavia During the War. *J. Gerontology*, 6: 102, 1951.

Herter, C. A.: An Experimental Study of the Toxic Properties of Indol. *N. Y. Med. J.*, 68: 89–93, 1898.

Hicks, C. S.: Studies in Tryptophane Feeding. *Austral. J. Exp. Biol. and Med. Sc.*, 3: 193–202, 1926.

Horwitz, A., L. A. Sachar, and K. Elman: Effects of Parenteral Amino Acids on the Growth of Rats. *Proc. Soc. Exp. Biol. and Med.*, 49: 118–121, 1942.

Houssay, F.: Etudes sur Six Générations de Poules Carnivores. *Arch. de Zool. Exp. et Génér.*, 6: 137–332, 1907.

Hueper, W. C., and C. T. Ichniowski: Hematic and Organic Reactions in Standardised and Graded Histamine Shock in Dogs. *J. Pharmacol. and Exp. Therap.*, 78: 127–138, 1943.

Hueper, W. C., and C. T. Ichniowski: Experimental Studies in Cardiovascular Pathology. VIII. Late Vascular Reaction of Histamine Shock in Dogs. *Amer. J. Path.*, 20: 211–216, 1944.

Hueper, W. C., and G. J. Martin: Tyrosine Poisoning in Rats. *Arch. Path.*, 35: 685–694, 1943.

Ingle, D. J., G. B. Ginther, and J. Nezamis: Effect of Diets in Rats on Adrenal Weights and on Survival Following Adrenalectomy. *Endocrinology*, 32: 410–414, 1943.

Ingle, L.: Effect of Environmental Conditions on Longevity. *Science*, 78: 511–513, 1933.

Jackson, H., and O. J. Moore. The Effect of High Protein Diets on the Remaining Kidney of Rats. *J. Clin. Invest.*, 5: 415–419, 1928.

Jackson, H., and M. D. Riggs: The Effect of High Protein Diets on the Kidneys of Rats. *J. Biol. Chem.*, 67: 101–107, 1926.

Klinger, B.: Beiträge zur Pharmacologischen Wirkung des Guanidins. *Arch. f. Exp. Path. und Pharmacol.*, 90: 129–141, 1921.

Koch, W. F.: On the Occurrence of Methyl-guanidine in the Urine of Parathyroidectomised Animals. *J. Biol. Chem.*, 12: 313–315, 1912.

Koch, W. F.: Toxic Bases in the Urine of Parathyroidectomised Dogs. *J. Biol. Chem.*, 15:43–63, 1913.

Koga, G.: Zur Therapie der Spontangangrän an den Extremitäten Dtsch. *Zschr. f. Chir.*, 121: 371–382, 1913.

Korenchevsky, V.: Contribution à l'étude Biologique du B. Perfringens et du B. Putrificus. *Ann. Pasteur*, 23: 91–95, 1909.

Korenchevsky, V.: Experimentelle Beiträge zur Lehre von der Gastro-Intestinalen Autointoxication. *Znblt. f. Bacteriol. Orig.*, 59: 526–549, 1911.

Korenchevsky, V.: The Problem of Aging: Basic Difficulties of Research. *Brit. Med. J.*, 1: 66–68, 1949.

Korenchevsky, V.: Endocrines and Aging. Age No Barrier. Report of N. Y. State Legisl. Comm., 120–128, New York, 1952.

Korenchevsky, V., S. K. Paris, and B. Benjamin: Effects of Castration and the Processes of Aging in Male Rats and Man. *J. Gerontology*, 8: 6–32, 1953.

Kutscher and Lohman: Nachweis Toxischer Basen im Harn. III. Mitteilung. *Zschr. f. Physiol. Chem.*, 49: 81–87, 1906.

Larson, E., and L. A. Elcourie: Tissue Changes in Parathyroid Tetany and in Guanidine Poisoning. *Amer J. Physiol.*, 85: 387–388, 1928.

Lewis, H. B.: Metabolism of Sulfur. *J. Biol. Chem.*, 65: 187–195, 1925.

Lignac, G. O. E.: Ueber Störung des Cystinstoffwechsels bei Kindern. *Arch. für Klin. Med.*, 145: 139–149, 1924.

Lignac. G. O. E.: A Syndrome of Affections of the Kidney, Stunted Growth, Rickets and Disturbed Cystine Metabolism. *Amer J. Med. Sc.*, 196: 542–547, 1938.

Lillie, R. D.: Histopathologic Changes Produced in Rats by the Addition to the Diet of Various Amino Acids. *U. S. Publ. Health Rep.*, 47: 83–93, 1932.

Lillie, R. D., L. L. Aschburn, W. H. Sebrell, F. S. Daft, and J. V. Lowry: Histogenesis and Repair of the Hepatic Cirrhosis in Rats Produced on Low Protein Diets and Preventable With Choline. *Publ. Health Rep.*, 57: 502–508, 1942.

Lillie, R. D., F. S. Daft, and W. H. Sebrell: Cirrhosis of the Liver in Rats on a Deficient Diet and the Effect of Alcohol. *Publ. Health Rep.* 50: 1255–1258, 1941.

Lindner, F., and R. Rigler: Ueber die Beeinflussung der Weite der Herzkranzgefässe durch Producte des Zellkernstoffwechsels. *Pflüg. Arch.*, 226: 697–708, 1931.

Lussana, F.: Azione Degli Aminoacidi Sopra il Circolo, la Pressione e la Secrezione Orinaria. *Arch. di Fisiol.*, 9: 307–317, 1910–1911.

Macht, D. I., and O. R. Hyndman: Relation Between the Chemical Structure of Bile Acids and Their Phytopharmacological and Zoopharmacological Reactions. *J. Pharmacol.*, 22: 483–490, 1924.

MacKay, E. M.: Factors Which Determine Renal Weight. XIV. The Relative Influence of Amino, Urea and Protein Nitrogen in the Diet. *J. Nutrition*, 6: 157–161, 1933.

Maclachlan, I., and N. B. Taylor: The Effect of Diet Upon Renal Hypertension. *Amer. J. Physiol.*, 129: 413, 1940.

Madden, S. C., J. C. Carter, A. A. Kattus, L. L. Miller, and G. K. Whipple: Ten Amino Acids Essential for Plasma Protein Production Effective Orally or Intravenously. *J. Exp. Med.*, 77: 277–295, 1943.

Madden, S. C., R. R. Woods, F. W. Shull, J. H. Remington, and G. H. Whipple: Tolerance to Amino Acid Mixtures and Casein Digests Given Intravenously. *J. Exp. Med.*, 81: 439–448, 1945.

Magnus, R.: Die Physiologische und Therapeutische Bedeutung des Cholins für die Magen-Darmtätigkeit. *Münch. Med. Wschr.*, 72: 249–254, 1925.

Major, R. H.: Relationship Between Certain Products of Metabolism and Arterial Hypertension. *J. Amer. Med. Asso.*, 83: 81–84, 1924.

Major, R. H., and W. Stephenson: Further Observations on the Elevation in Blood Pressure Produced by Guanidine Compounds. *John's Hopk. Hosp. Bull.*, 35: 186–187, 1924.

McCay, C. M., W. E. Dilley, and M. F. Crowell: Growth Rates of Brook Trout upon Purified Rations, Dry Skim Milk Diets, and Feed Combinations of Cereal Grains. *J. Nutrition*, 1: 233–245, 1929.

McCay, C. M.: Chemical Aspects of Aging and the Effect of Diet upon Aging. In Cowdry's *Problems of Aging*, 681–727.

Medawar, P. B.: Growth and New Growth. *Proc. Royal Soc. Med.*, 35: 590–595, 1942.

Medes, G.: A New Error of Tyrosine Metabolism: Tysosinosis. The Intermediary Metabolism of Tyrosine and Phenilalanine. *Biochem. J.*, 26: 917–940, 1932.

Merrill, J. P., S. Smith, E. J. Callahan, and G. W. Thorn: The Use of an Artificial Kidney. II. Clinical Experience. *J. Clin. Invest.*, 29: 425–438, 1950.

Metchnikoff, E.: The Prolongation of Life. W. Heinemann, London, 1907.

Metchnikoff, E.: Sur les Microbes de la Putréfaction Intestinale. *C. R. Acad. Sc.*, 147: 579–582, Paris, 1908.

Metchnikoff, E.: Etudes sur la Flore Intestinale. *Ann. Inst. Pasteur*, 22: 929–955, 1908.

Meyer, W.: The Conservative Treatment of Gangrene of the Extremities Due to Thrombo-anglitis Obliterans. *Ann. Surg.*, 63: 280–296, 1916.

Miller, A. J.: The Influence of High Protein Diet on the Kidneys. *J. Exp. Med.*, 42: 897–904, 1925.

Miller, E. C., and C. A. Baumann: Relative Effects of Casein and Tryptophane on the Health and Xanthurenic Acid Excretion of Pyridoxine-deficient Mice. *J. Biol. Chem.*, 157: 551–562, 1945.

Mitchell, P. H.: *Textbook of Biochemistry*. 2nd Edition. McGraw-Hill Co., Inc., New York, 1950.

Morehead, R. P., W. H. Fishman, and C. Arton: The Nephrotoxic Action of dl-serine as Related to Certain Dietary Factors. *Amer. J. Path.*, 22: 385–391, 1946.

Newburgh, L. H.: Production of Bright's Disease by Feeding High Protein Diets. *Arch. Int. Med.*, 24: 359–377, 1919.

Newburgh, L. H., and S. Clarkson: Renal Injury Produced in Rabbits by Diets Containing Meat. *Arch. Int. Med.*, 32: 850–869, 1923.

Newburgh, L. H., and A. C. Curtis: Production of Renal Injury in the White Rat by the Protein of the Diet. *Arch. Int. Med.*, 42: 801–821, 1928.

Newburgh, L. H., and P. L. Marsh: Renal Injuries by Amino Acids. *Arch. Intern. Med.* 36: 682–711, 1925.

Orla-Jensen, S., E. Olsen, and T. Geill: Senility and Intestinal Flora. *Kongel. Danske Videnskab. Selskab, Biolog. Skrifter*, 3: 1–38, 1945.

Osborne, T. B., L. B. Mendel, E. A. Park, and M. C. Winternitz: Physiological Effects of Diets Unusually Rich in Protein or Inorganic Salts. *J. Biol. Chem.*, 71: 317–350, 1926–27.

Park, W. H., and A. W. Williams: *Pathogenic Microorganisms*. 11th Edition, 230. Bailliere, Tindall and Cox. London, 1939.

Paton, N.: The Significance of Guanidine in the Animal Body. *Glasgow Med. J.*, 104: 297–314, 1925.

Paul, C. B.: On the Influence of the Excessive Meat Diet on the Male Reproductive Organs. *J. Physiol.*, 34: (Proceed.): XIV–XV, 1906.

Pearl, R.: *The Biology of Death*. J. B. Lippincott Co., Philadelphia, 1922.

Pezard, A.: Castration Alimentaire chez les Coqs, Soumis au Régime Carné Exclusif. *C. R. Acad. Sc.*, 169: 1177–1179, 1919.

Philipson, H., L. N. Katz, and S. Rodbard: The Effects of High-protein Diets on Experimental Renal Hypertension. *J. Exp. Med.*, 74: 591–600, 1941.

Polvogt, L. M., E. V. McCollum, and N. Simmonds: The Production of Kidney Lesions in Rats by Diets Defective Only in that They Contained Excessive Amounts of Proteins. *Bull. J. Hopk. Hosp.*, 34: 168–172, 1923.

Raida, H., and H. Liegmann: Epithelkörperchentetanie und Guanidin. *Zschr. f. ges. Exp. Med.*, 41: 358–362, 1924.

Reader, V., and J. C. Drummond: Relation Between Vitamin B and Protein in the Diet of Growing Rats. *Biochem. J.*, 20: 1256–1263, 1926.

Rettger, L. F., M. N. Levy, L. Weinstein, and J. E. Weiss: Lactobacillus Acidophilus and Its Therapeutic Application. Yale University Press, New Haven, 1935.

Rose, W. C.: The Nephropatic Action of the Dicarboxylic Acids and their Derivatives. *J. Pharmacol.*, 24: 147–157, 1924.

Rovighi, M.: De l'action des Produits Toxiques de la Putréfaction Intestinale sur le Foie et sur les Reins. *Semaine Med.*, 15: 466–467, 1895.

Rudzinska, M. A.: Overfeeding and Life Span in Toxophria Infusionum. *J. Gerontology*, 7: 544–548, 1952.

Rusch, H. P., B. E. Kline, and C. A. Baumann: The Influence of Caloric Restriction and of Dietary Fat on Tumor Formation With Ultraviolet Radiation. *Cancer Res.*, 5: 431–435, 1945.

Salant, W., and N. Kleitman: The Toxicity of Scatol. *J. Pharmacol.*, 19: 307–313, 1922.

Salkowski, E.: Ueber die Entstehung der Schwefelsäure und das Verhalten des Taurins im Thierischen Organismus. *Virch. Arch.*, 58: 460–508, 1873.

Samuels, S. S.: *Management of Peripheral Arterial Diseases.* Oxford University Press, New York, 1950.

Sarett, H. P., J. R. Klein, and W. A. Perlzweig: The Effect of the Level of Protein Intake Upon the Urinary Excretion of Riboflavin and Nicotinic Acid in Dogs and Rats. *J. Nutrition*, 24: 295–305, 1942.

Sauberlich, H. E., and C. A. Baumann: The Effect of Dietary Protein Upon Amino Acids Excretion by Rats and Mice. *J. Biol. Chem.*, 166: 417–428, 1946.

Saxton, J. A.: Nutrition and Growth and Their Influence on Longevity in Rats. *Biolog. Symp.*, 11: 177–196, 1945.

Saxton, J. A., and G. C. Kimball: Relation of Nephrosis and Other Diseases of Albino Rats to Age and to Modification of Diet. *Arch. Path.*, 32; 951–965, 1941.

Saxton, J. A., G. A. Sperling, L. L. Barnes, and C. M. McCay: Pathologic Studies of Rats Fed Different Amounts of Fluid Throughout Life. *J. Gerontology*, 8: 255–263, 1953.

Schaefer, A. E., D. H. Copeland, W. D. Salmon, and O. M. Hale: The influence of Riboflavin, Pyridoxine, Inositol and Protein Depletion-Repletion Upon the Induction of Neoplasms by Choline Deficiency. *Cancer Res.*, 10: 786–792, 1950.

Schliephake, E.: Zur Kenntniss der Cholinwirkung auf die Menschlichen Blutkreislauf. *Dtsch. Med. Wschr.*, 152: 113–135, 1926.

Schmidt, C. L. H., E. Adeburg, and T. Watson: On the Elimination of Taurin Administered to Man. *J. Biol Chem.*, 33: 501–503, 1918.

Selye, H., and H. Stone: Influence of the Diet upon the Nephrosclerosis, Periarteritis Nodosa and Cardiac Lesions Produced by the "Endocrine Kidney." *Endocrinology*, 43: 21–29, 1948.

Shoch, D., and S. J. Fogelson: The Effect of a Protein Denaturant on Histamine Ulcers. *Quart. Bull. Northwest. Univ. Med. School*, 16: 142, 1942.

Silbert, S.: Thrombo-angiitis Obliterans. *Surg. Gynec. and Obst.*, 61: 214–222, 1935.

Smith, A. H., and T. S. Moise: The Age Factor in the Response of the Rat to Level Dietary Protein. *J. Nutrition*, 4: 261–265, 1931.

Smith, A. H., T. S. Moise, and M. H. Jones: Diet and Tissue Growth. VI. Relation of Age to Renal Injury on Diet Rich in Protein. *Proc. Soc. Exp. Biol. and Med.*, 24: 746–748, 1927.

Sollmann, T.: *A Manual of Pharmacology.* 7th Edit., 354–356. W. B. Saunders Co., Philadelphia and London, 1948.

Spatz, H., and E. Wiechmann: Beobachtungen über den Einfluss des Cholins auf den Magendarmkanal beim Menschen. *Müunch. Med. Wschr.*, 71: 1425–1427, 1924.

Squier, T. L., and L. H. Newburgh: Renal Irritation in Man From High Protein Diet. *Arch. Intern. Med.*, 28: 1–19, 1921.

Sullivan, M. X., W. C. Hess, and W. H. Serbell: Studies on the Biochemistry of Sulphur. XII. Preliminary Studies on Amino Acids Toxicity and Amino Acids Balance. *U. S. Publ. Health Rep.*, 47: 75–83, 1932.

Tannenbaum, A.: Relationship of Bodyweight to Cancer Incidence. *Arch. Path.*, 30: 509–517, 1940.

Tannenbaum, A.: The Genesis and Growth of Tumors. II. Effects of Caloric Restriction per se. *Cancer Res.*, 2: 460–467, 1942.

Visscher, M. B., B. Zelda, R. H. Barnes, and I. Siverstein: The Influence of Caloric Restriction Upon the Incidence of Spontaneous Mammary Carcinoma of Mice. *Surgery*, 11: 48–53, 1952.

Watanabe, C. R.: Studies in the Metabolic Changes Induced by Administration of Guanidine Bases. *J. Biol. Chem.* 36: 531–545, 1918.

Weitzmann, J.: Der Einfluss von Serum and Plasma jünger und älter Menschen auf das Wachstum Menschlichen Embryonaler Gewebskulturen. *Zschr. f. Alterforschung.*, 2: 81–87, 1940.

White, F. R., J. White, G. B. Mider, M. G. Kelly, and P. W. Heston: Effect of Caloric Restriction on Mammary Tumor Formation in Strain C3H Mice and on the Response of Strain DBA to Painting with Methylchloranthrene. *J. Nation. Cancer Inst.*, 5: 43–48, 1944.

Zander, H. L., and N. B. Dreyer: Presence of a Depressor Substance in Essential Hypertension. *Arch. Intern. Pharmacol.*, 81: 245–250, 1950.

Editorial Note: Editorial expediency required curtailment of many textual references. However, the references given are pertinent to the discussion, and are as full as possible to the time of the submission of the manuscript.

NOTES ON THE SUBFAMILY RHADINOPSYLLINAE WAGNER 1930 (SIPHONAPTERA, HYSTRICHOPSYLLIDAE), AND DESCRIPTION OF A NEW SPECIES, *RHADINOPSYLLA MULTIDENTICULATUS**

HARVEY B. MORLAN† AND FRANK M. PRINCE‡

Information on fleas of western United States, obtained by extensive collections of the San Francisco Field Station (formerly Plague Suppressive Measures Laboratory), has been supplemented during the past three years with data from an ecology study of rodents and their ectoparasites at the Santa Fe Field Station. Clarification of the generic status of a new species, described in the present article, has required a consideration of other closely related Nearctic species of Rhadinopsyllinae.

Jellison *et al.* (1953, *Jour. Parasit.* 39 (6): 613) list North American genera of Rhadinopsyllinae as follows:

Actenophthalmus Fox 1925
Micropsylla Dunn and Parker 1923
Paratyphloceras Ewing 1940
Rectofrontia Wagner and Argyropulo 1934
Trichopsylloides Ewing 1938

Paratyphloceras is easily distinguishable from other genera of North American Rhadinopsyllinae by possession of a labial palpus with eight segments; *Trichopsylloides* is distinguished by the lack of more than a vestige of a genal ctenidium. The other three groups appear to be of doubtful validity. Hopkins (1952, *Jour Wash. Acad. Sci.* 42 (11): 365) listed *Actenophthalmus* and *Micropsylla* as subgenera of *Rectofrontia.* Traub (1953, *Jour. Wash. Acad. Sci.* 43 (3): 84) considers *Actenophthalmus* and *Micropsylla* to be synonyms or at best subgenera of *Rectofrontia.*

Dunn and Parker (1923, *Pub. Health Rep.* 38 (47): 2767) described *Micropsylla* as "near *Neopsylla* and *Rhadinopsylla*, differing from the former mainly by having four pairs of spines on each fifth tarsal segment, and from the latter by the overlapping of some of the genal spines." Hubbard (1947,

* From the Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare, Atlanta, Ga. Received for publication August 5, 1954.

† Currently assigned to Technical Development Laboratories, P. O. Box 769, Savannah, Ga.

‡ San Francisco Field Station, 15th Ave. and Lake Street, Building 19, San Francisco 18, California.

Fleas of Western North America: 348) after examination of 75 specimens stated that the genal teeth were not overlapping. Holland (1949, *Canada Dept. of Agric. Tech. Bull.* 70:89) states that in *Micropsylla* the metanotum is not divided by a transverse ridge.

Fox (1925, *Ent. News* 36: 121) described *Actenophthalmus* as "very close to *Rhadinopsylla* Jordan and Roths. (1912) but easily distinguished by the 6 pairs of lateral plantar bristles on the fifth tarsal segments of all legs." Fox also gave specific numbers of apical spines on abdominal tergites II through V. The present study indicates that the number of lateral plantar setae on tarsi V varies even in a single specimen and that there is individual variation in the number of apical spinelets on the abdominal tergites. These characters are certainly not of generic importance.

Wagner and Argyropula (1934, *Zeitsch. f. Parasitenk.* 7: 230) proposed the genus *Rectofrontia* and designated as genotype the palearctic species, *Typhlopsylla pentacanthus* Rothschild 1897. The authors have not examined the genotype but failed to find any characters in the description by Wagner and Argyropula by which *Rectofrontia* may be distinguished from the previously proposed genus *Actenophthalmus*. Costa Lima and Hathaway, in their *Fleas of the World* (Monografias do Instituto Oswaldo Cruz, No. 4, Dezembro, 1946), list 17 species of the genus *Rectofrontia* of which *fraterna* is the only North American representative. While unable to assess the generic placement of the 16 other species, the authors have examined specimens of Baker's *fraterna* and are of the opinion that it is cogeneric with *Acetenophthalmus* and *Micropsylla*.

Jordan and Rothschild (1912, *Nov. Zool.* 19: 367) described the genus *Rhadinopsylla* as follows:

"♂ ♀. Frons without tubercle, or this quite external, not placed in a groove. Antennal groove completely closed above, there being no sulcus across the vertex and the internal incrassation only being vestigial. A genal comb of five spines. Eye barely traceable. Labial palpi with five segments, the last segment posteriorly with a curved apical bristle as in true *Ctenophthalmus*. Pronotum with comb. Episternum of metathorax prolonged downwards, hind edge of sternum shortened; epimerum of metathorax narrower and dorsally more rounded than in *Ctenophthalmus*, very densely striated above the ventral margin; its stigma much more frontal than in the allied forms, being placed nearly half-way between the oblique upper edge and the anterior edge of the metepimerum. Metanotum without the short strong apical spines found on the proximal abdominal tergites, but with minute teeth. No antepygidial bristles in the ♂, but two on each side in ♀, both being long. Pygidium strongly convex in both sexes. Legs slender, particularly the femora. Basal internal rod of mid-coxa broad, bearing a mesial carina and therefore somewhat recalling a shoulder-blade, the corresponding rod of the hind-coxa narrow and quite short. Hind-coxa with a patch of short spiniform bristles on the inner surface. The fifth segment of all the tarsi with four pairs of lateral bristles as in the hind-tarsus of true *Neopsylla*."

Consideration of priority and available information on North American species of *Actenophthalmus*, *Micropsylla*, *and Recto-*

frontia leads to the conclusion that these forms are cogeneric with, and are herein tentatively proposed as subgenera of, *Rhadinopsylla.* The close relationship is especially noticeable in a comparison of male modified abdominal segments. With respect to the number of lateral plantar setae; the new species (described below) stands between and tends to connect Baker's *fraterna* and McCoy's *heiseri* (genotype of *Actenophthalmus*). To accommodate the North American species, the diagnosis of the genus *Rhadinopsylla* may be revised as follows:

Eye barely traceable or absent; genal comb usually with five spines on each side, the middle three being longest, occasionally with three to six spines on a side; head not divided above antennal fossae; labial palpus of five segments, the last segment posteriorly with a curved apical seta; pronotal comb with 14 to 21 stout spines; metepimerum with striarium, anterior terga each with two or more rows of setae; apical spinelets present at least on abdominal tergites II through V; female with two antepygidial setae on each side, these setae lacking in male; pygidium convex; a patch of thin spinelets on inner surface of coxa III; lateral plantar setae of tarsi V vary from four to seven on a side; spermatheca single.

Rhandinopsylla (Actenophthalmus) heiseri (McCoy)

Ctenophthalmus heiseri McCoy, 1911, *Ent. News* 22: 445.

Ctenophthalmus heiseri C. Fox, 1914, *Hyg. Lab. Bull.* No. 97: 24.

Actenophthalmus heiseri C. Fox, 1925, *Ent. News* 36: 121.

Actenophthalmus heiseri Jellison & Good, 1942, *Nat. Inst. Health Bull.* 178: 17.

Actenophthalmus heiseri Ewing & Fox, 1943, *U.S. Dept. Agric. Misc. Pub.* 500: 79.

Actenophthalmus heiseri Hubbard, 1947, *Fleas of Western North America.* Iowa State College Press: 352.

Actenophthalmus heiseri Hubbard, 1949, *Bull. S. Calif. Acad. Sci.*, 48: 122–124.

Morphological characters of apparent diagnostic value are given in the following supplemental description; observations are based upon examination of seven males and 15 females collected during November 1936 from *Citellus leucurus* taken in Inyo County, California.

Tarsi V (Fig. 13) most frequently with six lateral plantar setae on each side, the number may vary from five to seven but in all specimens some tarsi V have six setae on one or both sides. Eighth tergum of male lacking apical spinelets, seventh tergum

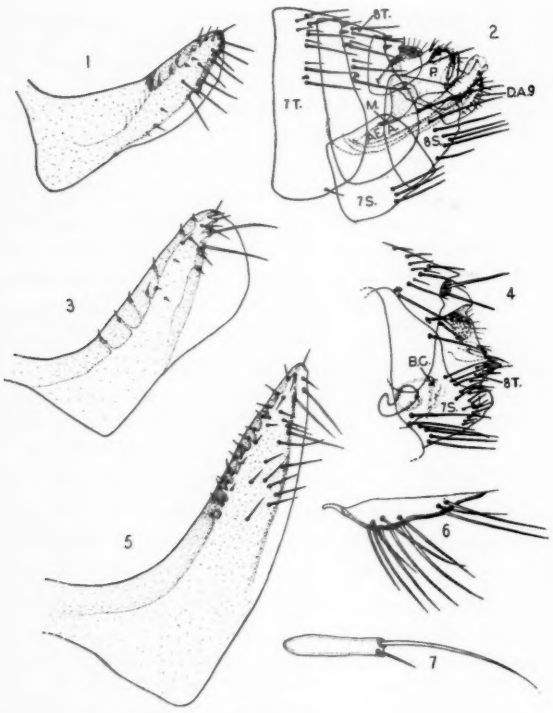


PLATE I

Rhadinopsylla (Actenophthalmus) multidenticulatus, new species.
FIG. 1. Distal arm of sternum IX.
FIG. 2. Modified abdominal segments, male.
FIG. 4. Modified abdominal segments, female.
FIG. 6. Ventral anal lobe, female.
FIG. 7. Anal stylet, female.

Rhadinopsylla (Rectofrontia) fraterna (Baker)
FIG. 3. Distal arm of sternum IX.

Rhadinopsylla (Actenophthalmus) heiseri (McCoy)
FIG. 5. Distal arm of sternum IX.

of female usually without apical spinelets. Apical spinelets, listed in order from terga II to VII with the most frequent number first and the range in parenthesis, for females are 6 (5–7), 6 (6–7), 5 (2–6), 4 (1–6), 2 (1–2), 0 (1–2); for males the numbers are 6 (6–7), 6 (6–7), 6 (5–6), 4 (2–4), and 2 (0–2) Pronotal ctenidium with 20 stout acuminate spines; from venter to dorsum, the spines become progressively longer. Posterior margin of mesonotum with three or four pseudosetae on each side. Immovable process of clasper (Fig. 12) rounded apically, ventral margin with strong concavity proximal to broadly rounded caudoventral angle, a heavily sclerotized projection above insertion of movable finger does not extend beyond caudal margin of clasper; movable finger about two times as long as broad. The distal arm of sternum IX (Fig. 5) is relatively long and narrow, with about 35 to 40 setae.

RHADINOPSYLLA (ACTENOPHTHALMUS) MULTIDENTICULATUS, n. sp.

The new species is similar to but varies from *R. heiseri* in the numbers of pronotal spines, lateral plantar setae, and apical spinelets on abdominal terga; males of the two species vary in the shape of the immovable process of the clasper, shape and setation of the distal arm of sternum IX, and relative width of the movable finger.

Head. Frontoclypeal margin angulate, gently rounded above but nearly vertical below the prominent tubercle. Preantennal area with four to six weak setae scattered between an upper row of five and a lower row of two longer setae. Eye absent. Genal ctenidium usually with five acuminate spines on each side, occasionally with four or six spines on a side. Considered in order from anterior to posterior, the fourth spine is slightly longer than the third, which is slightly longer than the second; these three spines are markedly longer than the subequal first and fifth spines. The first three spines are placed in a slightly oblique row, the fourth is inserted dorsocaudad of the third, the fifth dorsocaudad of the fourth spine. Spines about equal in width, the fifth only slightly heavier than the first.

Maxillary lobe extending to near apex of third segment of maxillary palpi. Labial palpi five-segmented, extending about three-fourths of length of fore coxae, last segment with curved seta at apical posterior margin. Scape of antenna with two to four small setae at base and two or three slightly larger median setae; pedicel with about six apical setae which do not reach the third segment of the clava. Anterior margin of antennal fossa heavily sclerotized and thickened above fifth genal spine; without hairs on dorsal margin. Postantennal region with two oblique rows of about five setae each and a marginal row of about five medium setae interspersed with weak setae, often with several additional weak setae scattered over the area.

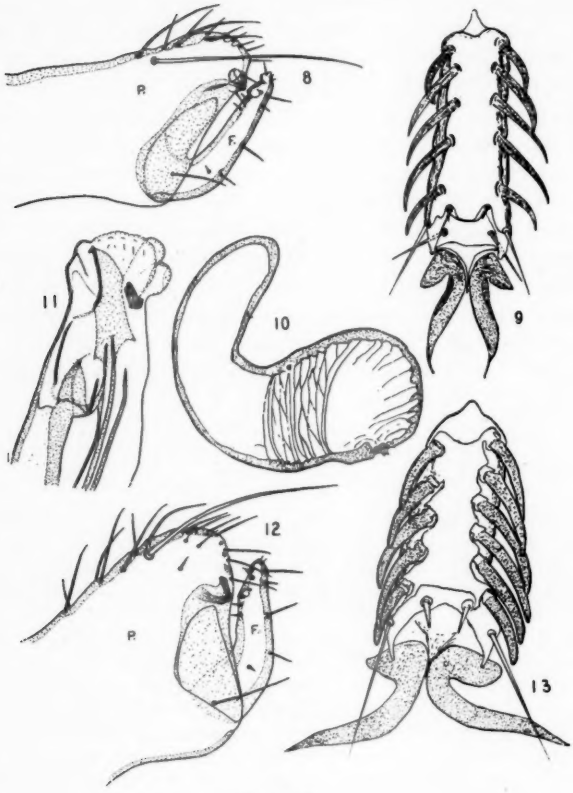


PLATE II

***Rhadinopsylla (Actenophthalmus) multidenticulatus*, new species.**

FIG. 8. Clasper with movable finger.
FIG. 9. Tarsus V.
FIG. 10. Spermatheca.
FIG. 11. Distal portion of aedeagus.

***Rhadinopsylla* (*Actenophthalmus*) *heiseri* (McCoy)**

FIG. 12. Clasper with movable finger.
FIG. 13. Tarsus V.

Thorax. Pronotum with a row of about 12 alternating large and small setae. Pronotal ctenidium usually with 16 stout acuminate spines, occasionally with 14 to 17; frequently with one or two additional smaller spines ventrally; from venter to dorsum the spines become progressively longer. Posterior

margin of mesonotum with about six large setae interspersed with small setae, the marginal row preceded by two or three rows of smaller scattered setae; one or two pseudosetae on each side.

Mesepisternum with a single large median seta. Mesepimere with two to four large setae. Metanotum with an anterior row of about five medium setae and a posterior row of about five larger setae alternated with small setae. Lateral metanotal area with a large and a small setae inserted along the well developed dorsal ridge. Metepisternum with one long and one small seta near posterior margin. Metepimere with three to five large setae, and a well developed striarium.

Legs. Procoxa with many long lateral setae scattered from base to apex. Outer basal surface of mesocoxa with about eight medium setae; of metacoxa with about 16 setae, in each case the distal setae are larger. Inner surface of metacoxa with about 20 spiniforms located just distad to middle on anterior margin. Outer surface of profemur with a medium seta at anterior apical margin, about three lateral setae, about 10 small setae along posterior margin in addition to a long heavy seta at the posterior apical margin. Mesocoxa and metacoxa each with a row of medium setae on the posterior margin, two setae on distal part of anterior margin, one long and one short heavy seta at the posterior apical angle. Tibiae with long heavy setae along posterior and apical margins, each tibia with an apical seta that extends beyond apex of the first tarsal segment. Metatarsus with distal seta of first segment extending beyond apex of second segment, seta of second segment extending beyond apex of fourth segment, and seta of third segment extending to apex of fourth segment. Fifth tarsal segment of each leg (Fig. 9) usually with five pairs of lateral plantar setae, occasionally one side with four or six setae.

Measurements (in microns) of holotype:

	Tibia	Tarsal Segment (exclusive of petiolate base) I	II	III	IV	V
Fore leg	106	49	67	49	39	112
Mid leg	235	120	112	67	46	116
Hind leg	322	252	175	95	74	140

Abdomen. Terga with anterior row of medium setae and posterior row of large setae interspersed with small fine setae. Apical spinelets present on terga II through VIII in males and terga II through VII in females. The number of apical spinelets, listed in order from terga II to VII or VIII with the most frequent number first and the range in parenthesis, for 23 females are 7 (5–8), 8 (6–11), 8 (6–9), 5 (4–6), 2 (2), 2 (2); for 22 males the numbers are 6 (6–10), 8 (6–12), 9 (6–12), 8 (6–9), 7 (4–8), 4 (4–7), and 4 (2–5). Basal sternum usually with one seta on ventral margin. Unmodified sterna usually with three of four setae on each side. Antepygidial setae lacking in male; female with two, dorsal seta slightly longer than ventral.

Modified Abdominal Segments, Male. (Figs. 1, 2, 8, 11). Eighth tergum nearly as large as tergum VII, with two heavy spinelets and about seven setae. Eighth sternum prominent extending dorsad slightly beyond apex of

proximal arm of ninth sternum and caudad beyond base of movable finger, posterior margin convex, ventral half with seven or eight long setae.

Immovable process of clasper (Fig. 8) with a very long seta beyond middle of dorsal margin, numerous smaller setae along dorsoapical margin, and a few tiny subapical setae. Dorsad of the insertion of the movable finger is a heavily sclerotized acuminate projection that extends beyond the concave posterior margin of the clasper; with a small thin acetabular seta inserted above movable finger. Ventral margin of clasper with weak concavity proximal to rounded caudoventral angle. Movable finger (Fig. 8), about three times as long as broad, apical two-thirds conical, tapering to a narrowly rounded apex, with three to five subapical setae, three small median setae on distal third and one on proximal third of finger, two marginal setae on distal half below projection on concave anterior margin, three longer setae evenly spaced on convex posterior margin. Manubrium (Fig. 2) broad, short, abruptly narrowed for a short distance before subacute apex. Ninth tergum large, extending ventrally below the apex of proximal arm of ninth sternum.

Ninth sternum with distal arm (Fig. 1) tapering to narrowly rounded apex, with about 25 setae inserted on distal half; concave caudoventral margin is lightly sclerotized and lacks marginal setae except at the apex. Aedeagal apodeme about twice as long as aedeagus proper (Figs. 2, 11); membranous hood with slightly sinuous convex apical margin; crochets apparently represented by irregular sclerotized basal portions and membranous, rectangular apical flaps.

Female. (Figs. 4, 6, 7, 10). Seventh sternum with broad, shallow sinus near ventral margin; with six anterior setae near ventral margin; posterior margin with about 15 medium setae ventrally and a longer seta inserted above the sinus. Eighth tergum with two medium setae near spiracle and about 12 smaller setae near caudal margin. Dorsal anal lobe with several marginal and submarginal setae along heavily sclerotized dorsal margin. Anal stylet (Fig. 7) about five times as long as broad; with a long apical dorsal seta and one or two small subapical ventral setae. Ventral anal lobe (Fig. 6) heavily sclerotized along ventral margin, with about five median and about six marginal setae. Spermatheca (Fig. 10) without definite demarcation between head and strongly recurved tail. Bursa copulatrix sinuous, with expanded apex and sclerotized ventral extension.

Holotype. Male (ex *Onychomys leucogaster melanophrys* Merriam, Airport, Santa Fe County, New Mexico, November 19, 1952, H. B. Morlan, collector) deposited in the U. S. National Museum No. 62395.

Allotype. Female (ex *Onychomys leucogaster melanophrys* Merriam, Airport, Santa Fe County, New Mexico, November 22, 1951, H. B. Morlan, collector) deposited in the U. S. National Museum.

Paratypes. Four males and three females with same collection data as holotype, one male and two females with same collection data as allotype; one male and one female each to U. S. Army Medical Service Graduate School, Washington, D. C. and U. S. Public Health Service laboratories as follows: Rocky Mountain Laboratory, Hamilton, Montana; Communicable Disease Center Insect Collection, Atlanta, Georgia; Communicable Disease Center,

San Francisco (California) Field Station; and Santa Fe (New Mexico) Field Station.

Records

R. multidenticulatus has been taken on a variety of rodents but occurs most frequently on the grasshopper mouse, *Onychomys leucogaster*. Of 78 specimens collected in Santa Fe County, New Mexico, 41 were from *O. leucogaster*, 12 from *Dipodomys ordii*, 15 from *Dipodomys spectabilis*, one from *Peromyscus leucopus*, three from *Neotoma micropus*, one from *Neotoma albigula*, four from nest of *N. albigula*, and one from nest of *D. spectabilis*. Also examined were 14 specimens taken in March 1948 by L. Williams from a *D. spectabilis* nest in Bernalillo County, New Mexico. Nests of *O. leucogaster* have not been examined.

Of 26 specimens collected during the past 15 years by the San Francisco Field Station, 21 were from *O. leucogaster*, one from *N. albigula*, one from *Neotoma cinerea*, two from *Neotoma* sp., and one from *D. ordii*. The San Francisco specimens include collection records in Texas from Cochran and Yoakum Counties; in New Mexico from Luna, Bernalillo, Grant, San Miguel, Hidalgo and Socorro Counties; in Arizona from Cochise, Yavapai, Greenlee, and Navajo Counties. The new flea is most prevalent from November through January and has not been collected during the months of June through September.

RHADINOPSYLLA (RECTOFRONTIA) FRATERNA (BAKER)

Typhlopsylla fraterna Baker, 1895, *Can. Ent.* 27: 189.
Ctenophthalmus fraternus Baker, 1904, *U. S. Nat. Mus. Proc.* 27: 423.
Rhadinopsylla fraterna Rothschild, 1913, *The Entomologist* 46: 297.
Rhadinospsylla fraternus, Jordan & Rothschild, 1923, *Ectoparasites* I: 314.
Neopsylla hamiltoni Dunn, 1923, *U. S. Pub. Health Repts.* 38: 2770.
Rectofrontia Wagner and Argyropulo, 1934, *Zeitsch, f. Parasitenk.* 7: 230.
Rectofrontia fraternus Wager, 1936, *Can. Ent.* 68: 203.
Rectofrontia fraterna Jordan, 1937, *Nov. Zool.* 40: 270.
Rectofrontia fraterna I. Fox, 1940, *Fleas of Eastern United States:* 38.
Rectofrontia fraterna Hubbard, 1947, *Fleas of Western North America:* 355.
Rectofrontia fraterna Holland, 1949, *Canada Dept. of Agriculture Tech. Bull.* 70: 91.
Rectofrontia fraterna Tipton, 1950, *Gt. Basin Nat.*, 10: 63.

Collections of the San Francisco Field Station include records of *R. fraterna* as follows: from *Citellus armatus* (Madison County, Montana); *Citellus beldingi* (Wallowa County, Oregon); *Citellus columbianus* (Beaverhead, Jefferson, and Powell Counties, Montana; Lincoln County, Washington); *Citellus franklini* (Rolette County, North Dakota); *Citellus richardsoni* (Beaverhead, Gallatin, Glacier, Madison, Toole, and Valley Counties, Montana; McIntosh and McLean Counties, North Dakota; Fremont and Natrona Counties, Wyoming); *Citelus washingtoni* (Adams County, Washington); *Cynomys gunnisoni* (Lake and Saguache Counties, Colorado); *Cynomys leucurus*

(Jackson County, Colorado; Big Horn, Hot Springs, Park, and Sublette Counties, Wyoming); *Cynomys ludovicianus* (Garfield County, Montana; Johnson County, Wyoming); *Eutamias amoenus* (Yellowstone National Park, Wyoming); *Eutamias merriami* (Monterey County, California); *Neotoma albigula* (Hayes County, Nebraska).

Only a single male of *R. fraterna* has been found during the extensive collections in Santa Fe County, New Mexico. It was collected from *Clethrionomys gapperi* taken in October 1953 at an elevation of between 10 and 11 thousand feet above sea level.

Diagnosis

R. fraterna, R. heiseri, and *R. multidenticulatus* are very similar; in each there is a well developed dorsal ridge of the lateral metanotal area which readily separates them from species of the subgenus *Micropsylla* that lack this transverse ridge in the lower portion of the metanotum.

Males of the three species are readily separated by the shape and setation of the distal arm of sternum IX (Figs. 1, 3, 5); in *R. heiseri* it is relatively long, with 35 to 40 setae; in *R. fraterna* and *R. multidenticulatus* it is shorter, with only about 25 setae; the greatly expanded caudoventral margin is distinctive in *R. fraterna.*

In *R. fraterna* and *R. heiseri,* the pronotal ctenidium contains about 20 spines; in *R. multidenticulatus* there are less than 20 (usually 16) spines.

In spite of variation in number of lateral plantar setae on a single tarsus V, individual specimens may be distinguished by determination of the most frequent number of setae on each side of each tarsus V; the most frequent number per side is four in *R. fraterna,* five in *R. multidenticulatus,* and six in *R. heiseri.*

Males of *R. multidenticulatus* have apical spinelets on tergum VIII; males of *R. heiseri* and *R. fraterna* lack spinelets on this segment. Females of *R. multidenticulatus* have two apical spinelets on tergum VII; females of *R. heiseri* and *R. fraterna* usually lack spinelets on tergum VII and tergum VI, respectively.

Acknowledgments

We are most grateful to Dr. Karl Jordan who, in 1949, examined and recognized a pair of our specimens as a new species near *Actenophthalmus.* We are also very pleased to acknowledge the helpful advice on generic status of the new species and related groups which was received in personal communications of January 1954 from C. H. E. Hopkins, Robert Traub, and G. P. Holland.

Abbreviations

AE.A., aedeagal apodeme; B. C., bursa copulatrix; D. A. 9, distal arm of ninth sternum; f., movable finger; M., manubrium; P., immovable process of clasper; 7 S., seventh sternum; 8 S., eighth sternum; 8 T., eighth tergum.

SANGUININ: LOCAL WOUND HEALING EFFECT ON CHRONIC CUTANEOUS ULCERATIONS*

SAMCHAI NILASENA, FENWICK L. WATTS, J. FRED MULLINS AND LUDWIK ANIGSTEIN

The healing of chronic, superficial ulcers, particularly of the lower extremities, is one of the most discouraging problems encountered by the practicing physician. Surgical intervention, such as excision, skin grafting, or amputation of the extremity, is frequently resorted to in such cases.

Human red cell discard from the processing of blood plasma during World War II was first used clinically at the New York Postgraduate Hospital (Moorhead and Unger, 1943). Since then human blood in the form of dried blood cells (Seldon and Young, 1943), red blood cell paste (Murray and Shaar, 1944), fresh human blood and concentrated plasma (Naide, 1943), blood cell gel (Kanski, 1946; Allgöwer, 1949) and other forms of blood (Orbach, 1946) have been used clinically for the treatment of chronic ulcers with encouraging results. Seldon and Young seemed very enthusiastic with their results; however, they expressed that considerable difficulty arises with obtaining fresh human blood. "At present the thought is entertained: Would it be possible to use the red blood cells from a source other than human blood? If this is possible, when the source of human blood cells drops off after this war, bovine cells might do an equally good job." (Seldon and Young, 1943).

Anigstein, Whitney, and Micks (1950) demonstrated an antibacterial factor in blood engorged cattle ticks, and in their subsequent work (Whitney, Anigstein, Micks, 1950) it was found that red blood cells of cattle possess antibacterial properties. Sanguinin, a product made from bovine red blood cells, showed in recent animal experimentation a suppressive effect on streptoccal infection in mice (Micks, Whitney, and Anigstein, 1951). Furthermore, debridement and healing effect was observed when Sanguinin was applied topically in open, contaminated, purulent wounds of the skin of dogs (Bader, 1952).

* From the Department of Dermatology and the Department of Preventive Medicine and Public Health, The University of Texas, Medical Branch, Galveston.

The results of these experiments were so encouraging that one of the authors (L. A.) suggested that Sanguinin be used in human patients by direct topical application of the dry powder to the surface of the wounds. This product was prepared in the Rickettsial Research Laboratory by Miss Dorothy Whitney according to the technical principles as previously described (1950, 1951).

Consequently a patient with extensive, chronic ulceration of the legs was selected for treatment with Sanguinin, as the recommended approach was that of amputation of the extremity. The technique for application of the Sanguinin was arbitrarily adopted, *i.e.*, dry powder to cover the area in a layer about 5 mm. in thickness with a dry bandage dressing to be changed every two to seven days, depending upon the amount of purulent discharge. The course of the patient in the hospital is as follows:

This patient was a 63-year-old colored male who had been seen in Surgery Clinic for approximately one year and throughout this period of time had been treated with penicillin and local therapy. He had not been to the Surgery Clinic for approximately three months and presented himself to the Dermatology Clinic on February 16, 1954. On his initial visit to the latter clinic he was found to have a very foul smelling, edematous ulceration of the left lower leg and foot. The ulceration extended over almost the entire pretibial region as well as the dorsum of the foot. Over the dorsum of the foot there was a thick eschar which covered the central portion of the lesion and was approximately 10 to 12 cms. in diameter. Cultures of these lesions revealed *Proteus morganii* and *Streptococcus hemolyticus*. After admission to the hospital, his therapy consisted of bed rest, potassium permanganate soaks, and neomycin lotion. In addition to this, he was found to have late latent syphilis and a low grade cystitis, for which penicillin and gantrisin were given respectively.

Saguinin was begun as local applications to the foot and leg on February 24, 1954. By June 1, 1954, the lesion on the dorsum of the left foot had decreased to 5 cm. in diameter, and the pretibial lesion was approximately 2.5 cm. in diameter. As demonstrated by the pictures herein, one can see the tremendous improvement with this therapy. With the lesions at this rather minimal size, it was felt that the patient should have skin grafts

to be followed by gelatin boots. This procedure was carried out, and the patient has subsequently done very well with no evidence of recurrence of his initial lesion.

Discussion

The appearance of the fresh, healthy, and clean granulation tissue as it has replaced the previous necrotic debris is evident from the photographs (Figs. 1, 2, 3) showing various phases

FIG. 1. The initial lesion showing severe necrosis involving the epidermis and dermis of the pretibial region as well as the thick eschar over the dorsum of the foot.

of the lesions during treatment with Sanguinin. This is apparently a characteristic feature of Sanguinin action on the healing process. This stimulation of the granulation tissue is accompanied by a regeneration of the epithelium and by the growth of the epithelial border from the edge of the ulcer, with a subsequent reduction of the ulcerative area. Although the mechanism of action of Sanguinin is still rather obscure, the direct application of this product may act as a nutritional factor to the diseased tissue. We are still unaware of the intrinsic characteristics of Sanguinin responsible for the luxuriant growth of granulation tissue and epithelialization. Although the antibacterial properties of Sanguinin cannot be ignored in this regard, the striking antienzymatic action of Sanguinin in lowering

the production of tissue catalase, as observed by Cline and associates (1951), may perhaps have some bearing on the wound healing process.

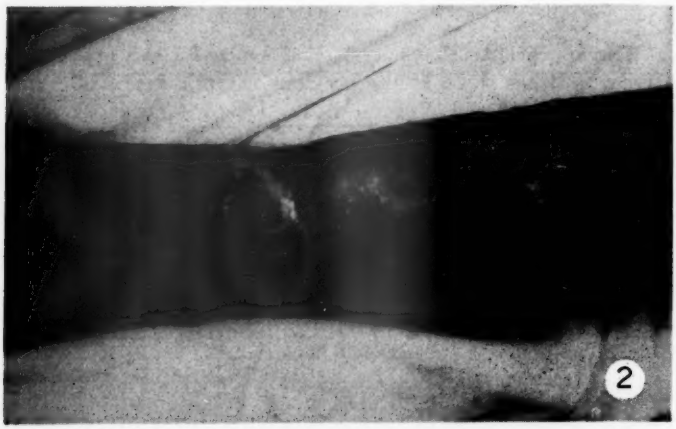


FIG. 2. This demonstrates the marked debridement which has occurred as well as the healthy granulation tissue.

FIG. 3. This photograph was taken six weeks after the institution of therapy and just previous to skin grafting.

Inasmuch as the patient who is presented here has responded satisfactorily to our treatment without amputation, a larger series is necessary to suitably evaluate the granulation tissue stimulating factor of Sanguinin. We have not lost sight of the fact that this patient was hospitalized for a considerable period of time, which was also an adjunct to the good result. At this time we wish to present this article as the first report on the use of Sanguinin in a human subject.

Conclusions. A case of a chronic and extensive ulceration of the leg treated locally with Sanguinin, a blood derivative, is presented.

The results of treatment with Sanguinin are encouraging enough to justify further investigation of this agent.[1]

REFERENCES

Anigstein, L., D. M. Whitney, and D. W. Micks: Antibacterial Factor in Tick Extracts. *Tex. Rep. Biol. and Med.*, 8: 86–100, 1950.

Allgöwer, M.: Verwendungs Moglichkeiten Blut und Bluthestandteilen in der Wundbehandlung, Helvet. *Chirurgica Acta*, 16: 249–255, 1949.

Bader, M. N.: Wound Healing Properties of the Blood Hydrolysate Sanguinin. *Tex. Rep. Biol. and Med.*, 10: 773–777, 1952.

Cline, J. K., R. B. Johnson, E. Scholz, and C. M. Welb: Studies on the Anti-Catalase-Producing Substances of Malignant Tumors. I. Products Related to Hemoglobin, the Action of Sanguinin. *Cancer Research*, 11: No. 4, 1951.

Kanski, J. G.: Blood Cell Jel for Chronic Surface Ulcers. *Am. J. Surg.*, 72: 580–582, 1946.

Moorhead, J. J., and L. G. Unger: Human Red Cell Concentrate for Surgical Dressings. *Am. J. Surg.*, 59: 104–105, 1943.

Micks, D. W., D. M. Whitney, and L. Anigstein: Effect of Sanguinin, an Antibacterial Agent from Blood, on Streptococcal Infection in Mice. *Proc. Soc. Exp. Biol. and Med.*, 78: 573–576, 1951.

Murray, C. F., and C. M. Shaar: Red Blood Cell Paste in Treatment of Ulcers. *J.A.M.A.*, 125: 779, 1944.

Naide, M.: Treatment of Leg Ulcers With Blood and Concentrated Plasma. *Am. J. Med. Sci.*, 205: 489–493, 1943.

Orbach, E. J.: The Use of Blood-Kaolin Penicillin Paste in the Treatment of Varicose and Torpid Leg Ulcers. *Am. J. Surg.*, 71: 253–255, 1946.

Seldon, T. H., and H. H. Young: Use of Dried Blood Cells in Wound Healing. *Proc. Staff Meet. Mayo Clin.*, 18: 385–389, 1943.

Whitney, D. M., L. Anigstein, and D. W. Micks: Antibacterial Activity of Hydrolyzed Red Blood Cells *In Vitro*, *Proc. Soc. Exp. Biol. and Med.*, 74: 346–350, 1950.

[1] After treating several more patients, it was found that the ulcers of arterial dysfunction or arteriosclerotic etiology respond more favorably than those due to venous abnormalities.

PROCEEDINGS, TEXAS BRANCH OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

MAY 28–29, 1954

MEMORIAL STUDENT CENTER, A&M COLLEGE OF TEXAS

1. Preliminary investigations of the mineral nutrition of *Clostridium botulinum*. L. E. Bowers and O. B. Williams, Department of Bacteriology, The University of Texas.

2. Oxidation-reduction potentials in cultures of *Clostridium botulinum*. A. I. Laskin and O. B. Williams, The Department of Bacteriology, The University of Texas.

3. Role of thermo treatment of phosphate-buffered glucose in germination of Clostridium spores at 75° C. L. I. Galyen and E. Staten Wynne, Division of Experimental Pathology, The University of Texas, M. D. Anderson Hospital for Cancer Research, Houston.

4. Nucleoprotein phosphate content of virulent and avirulent *Bacillus anthracis* and *Bacillus cereus* spores. E. O. Bennett and R. P. Williams, Department of Biology, University of Houston and Department of Microbiology, Baylor Medical School, Houston.

5. Survival times of bacteria in heavy and emulsion type cutting oils. H. O. Wheeler and E. O. Bennett, Department of Biology, University of Houston, Houston.

6. Effect of induced aureomycin resistance on the removal of vitamin B_{12} from culture medium by an intestinal isolate *Escherichia coli*. Glen E. Peterson, Department of Biology, University of Houston, Houston.

7. Stimulation of soil microorganisms by antibiotics. R. J. Hervey, Texas Agricultural Experiment Station, Temple.

8. Some aspects of cellulose decomposition. T. P. O'Barr, Orville Wyss, and V. L. Frampton, Department of Bacteriology, The University of Texas, Austin.

9. Motile colonies of *Bacillus alvei* (?). W. L. Tidwell, Department of Biology, A. & M. College, College Station.

10. Infectious synovitis of chickens. F. K. Wills, Department of Veterinary Research, Texas Agricultural Experiment Station, College Station.

I. M. LEWIS LECTURE

DR. PAUL S. PRICKETT

Mead Johnson & Company

Evansville, Indiana

"Influence of the U. S. Pure Food and Drug Laws on Food Microbiology"

11. Diphtheria toxin-antitoxin reactions within the "equivalence" zone. E. B. Blair and V. T. Schuhardt, Pathological Microbiology Laboratory,

Clayton Foundation and Department of Bacteriology, The University of Texas, Austin.

12. The plate complement fixation test with leptospiral antigens. R. M. Pike, H. B. Owens, and Winona Humes, Department of Microbiology, Southwestern Medical School, The University of Texas, Dallas.

13. Immunologic effects of homologous fetal antigen on tumor susceptibility. J. D. Flintjer and R. B. Mefferd, Jr., Southwest Foundation for Research and Education, San Antonio.

14. A black *Candida* isolated from a case of Hodgkin's disease. E. Staten Wynne and Cora L. Gott, Division of Experimental Pathology, The University of Texas, M. D. Anderson Hospital for Cancer Research, Houston.

15. Some aspects of the bat rabies problem in Texas. J. E. Grimes, Thelma D. Sullivan, and J. V. Irons, Texas State Department of Health, Austin.

16. Recent findings on ornithosis as an occupational disease in poultry dressing establishments. J. V. Irons and Thelma D. Sullivan, Texas State Department of Health, Austin.

17. Alteration in susceptibility to mice to Coxsackie virus infection by use of foster mothers. S. E. Sulkin, A. Behbehani, and H. C. Wallis, Department of Microbiology, Southwestern Medical School, The University of Texas, Dallas.

18. The Effect of Cortisone on the Rickettsemia Pattern of Typhus Infected Rats. Morris Pollard, Department of Public Health and Preventive Medicine, The University of Texas, Medical Branch, Galveston.

19. *In vitro* effects of Diamox on sulfonamide and antibiotic activity. F. B. Engley, Jr., Rose Marie Steeg, and Arthur Ruskin, Departments of Bacteriology and Medicine, The University of Texas, Medical Branch, Galveston.

20. Inhibitory effects of salicylate derivatives against fungi. J. B. Nash, J. K. Schoening, and J. D. Archer, Department of Pharmacology and Toxicology, The University of Texas, Medical Branch, Galveston.

21. Microorganisms in acid coal mine waters. Kenneth Temple, The Texas Company, Bellaire.

Role of Thermal Treatment of Phosphate-Buffered Glucose in Germination of Clostridium Spores at 75° C. L. I. Galyen and E. Staten Wynne, Division of Experimental Pathology, The University of Texas, M. D. Anderson Hospital and Tumor Institute, Houston.

In a medium of 2 per cent glucose and 0.04 M phosphate (mixture of KH_2PO_4 and K_2HPO_4) autoclaved together for 80 minutes at 121° C., a rapid loss of heat stability was consistently observed at 75° C. with spores of *Clostridium botulinum* 62A, *Clostridium botulinum* 115B, *Clostridium feseri*, *Clostridium perfringens*, and putrefactive anaerobe No. 3679. Evidence is presented that this decrease in heat stable spores results from actual germi-

nation. Germination was 90 per cent or more with the *C. botulinum* strains after 2 hours at 75° C., and was virtually 100 per cent with the other 3 strains after 1 hour. Kinetic data secured with *C. perfringens* and P.A. 3679 indicated a short lag period followed by logarithmic germination. With *C. perfringens*, the extent of germination was shown to depend on the length of time of autoclaving glucose and phosphate together. Vegetative cells of all 5 test species failed to grow at 50° C. Germination at 75° C. may differ markedly from germination at lower temperatures since germination at the higher temperature is much faster than would be predicted from kinetic data for complex media at lower temperatures.

Germination at 75° C. of spores whosse corresponding vegetative cells are manifestly mesophilic is considered another line of evidence in favor of the concept of Wynne (*Bact. Rev.*, 16: 101–110, 1952) that spore germination is fundamentally different from subsequent vegetative cell development.

Stimulation of Soil Microorganisms by Antibiotics. Ralph J. Hervey, Substation No. 5, Texas Agricultural Experiment Station, Temple.

Antibiotics in low concentration appear to stimulate plant growth in soils. This preliminary study was undertaken to determine possible microbial factors involved.

Population counts, nodule counts and nitrate concentrations were determined in fallow and cropped soil watered periodically (5 times over a period of 35 days) with weak solutions of four antibiotics (10–50 p.p.m.) and water.

The general effect of the antibiotics was to increase microbial populations, in some cases markedly, over those in water-treated soils. Fallow soil populations reacted somewhat differently from cropped-soil populations. The greatest stimulation to bacterial and actinomycete populations occurred during the early antibiotic applications. Thiolutin (25 p.p.m.) induced a total population of 167 million per gram. Water alone produced 48 million. After 35 days many populations of antibiotic-treated soils dropped below that of water-treated soil.

Antibiotic-treated, cropped-soil populations, in contrast, remained relatively high throughout the period of treatment. Five

applications of actidione (10 p.p.m.) induced a total population of 58 million. Water alone produced 29 million.

Rhizobia populations apparently were increased by antibiotics. Bacitracin induced 9 nodules per plant against 2 per plant for the control.

The activity of nitrifying bacteria apparently was stimulated by actidione, but inhibited by thiolutin.

Some Aspeccts of Cellulose Decomposition by Myrothecium verrucaria. T. P. O'Barr, Orville Wyss, and V. L. Frampton.

In this study attention has been directed toward the cellulosic residue remaining after extended growth of *Myrothecium verrucaria.* It was believed that this approach might yield a clearer picture concerning the pathway of cellulose decomposition by this organism than has hitherto been obtained on the basis of enzyme studies.

Attacked, non degraded cellulose, free from adhering mycelium, may be obtained for study through preparation of the cellulose triacetate. On the basis of the reducing power, saponification number and acetyl number of specimens so obtained, it would appear that extensive action of a diffusable cellulose does not occur.

Moreover, viscometric and diffusimetric data further strengthen the interpretation that no discernable decrease in the degree of polymerization of the cellulose specimen results as the result of fungal attack. Therefore, it is proposed that the process is essentially localized in nature with respect to growth of the fungus.

Infectious Synovitis of Chickens. F. K. Wills, Department of Veterinary Bacteriology and Hygiene, A. and M. College of Texas.

Studies are reported on an agent which produces a subacute or chronic disease of chickens characterized by purulent or caseous exudate in synovial tissues. The exact nature of the causative agent has not been determined—investigations suggest a large particle virus, pleuropneumonia-like organism, or other small bacteria.

The disease has been transmitted by intravenous, intramuscular, subcutaneous, and air sac inoculations. Transmission

by direct contact was observed in only one instance. The presence of the agent in blood and plasma, liver and spleen, and joint and bursa exudates has been determined.

The agent has been passed serially five times in the allantoic cavity of chick embryos.

Effect of Induced Aureomycin-resistance on the Removal of Vitamin B_{12} from Culture Medium by an Intestinal Isolate of Escherichia coli. G. E. Peterson, Department of Biology, University of Houston.

Serial transfers in aureomycin-containing broth caused about a 70-fold increase in the drug-resistance of a strain of *Escherichia coli*. Inocula of the parent and the resistant organisms were cultured at 37° C. for 24 hours in a synthetic glucose-mineral salts basal medium (I) and the following modifications thereof: basal + 5 mμg. B_{12}/ml. (II), basal + 0.3 μg. aureomycin/ml. (III), basal + 5 mμg. B_{12}/ml. + 0.3 μg. aureomycin/ml. (IV) and basal + 5 mμg. B_{12}/ml. + 25 μg. aureomycin/ml. (V). After separation from the culture supernates the cells were dried, weighed, resuspended and digested under toluene with trypsin for 24 hours at 37° C. The digested cells and their respective culture supernates were then tested for vitamin B_{12} by the microbiological assay which employs E. coli (113–3).

Vitamin B_{12} could not be detected in either the cells or the filtrates of cultures from (I) and (III). Both organisms caused most of the added vitamin to disappear from mediums (II) and (IV), however, following digestion with trypsin, the cells of the resistant organism yielded a larger concentration of the vitamin than did those of the parent. The resistant variant removed very little B_{12} from (V) and that which was taken up was almost quantitatively liberated from the cells by trypsin digestion. The parent strain failed to grow in (V).

These preliminary studies may explain reports that the vitamin B_{12}-sparing effect of dietary aureomycin in certain animals is accompanied by an increase in the "free" B_{12} content of the intestines and an enhancement of aureomycin-resistance within certain members of the intestinal microflora.

Comparative Nucleoprotein Phosphorus Content of Vegetative Cells and Spores of Bacillus anthracis, virulent and avirulent, and Bacillus cereus. E. O. Bennett and R. P. Williams,

Department of Biology, University of Houston and Department of Microbiology, Baylor University College of Medicine, Houston.

Twenty *Bacillus anthracis* strains (nine virulent; eleven avirulent) and eight *Bacillus cereus* strains were analyzed for the nucleoprotein phosphorus content of their vegetative cells and spores. Eighteen hour cultures were used for vegetative cells and five day cultures for spores. Nucleoprotein content was determined by the Schmidt and Thannhauser method. The results showed that in every case the total nucleoprotein content, measured as micrograms of phosphorus per milligram dry weight of cells, was less in spores than in vegetative cells. Vegetative cells of *B. anthracis*, both virulent and avirulent, had twice the nucleoprotein content of spores, while vegetative cells of *B. cereus* had one-third more nucleoprotein than spores. Pentose-nucleic acid (PNA) content of all strains was similar, but spores contained only half as much vegetative cells. Virulent and avirulent *B. anthracis* spores contained similar amounts of desoxypentosenucleic acid (DNA), but vegetative cells of virulent organisms contained twice as much DNA as avirulent cells. Spores of *B. cereus* had two-thirds more DNA than vegetative cells. The PNA/DNA ratios of vegetative cells and spores of virulent *B. anthracis* were similar, but PNA/DNA ratios of avirulent *B. anthracis* and *B. cereus* spores were markedly lower than the ratios of vegetative cells.

Survival Times of Bacteria in Heavy and Emulsion Type Cutting Oils. H. O. Wheeler and E. O. Bennett, Department of Biology, University of Houston.

Commercial cutting oils are heavily contaminated with bacteria which may lead to degradation of these oils and effect the health of workers who come in contact with them. This study was undertaken to ascertain the survival times of different bacteria in cutting oils in order to determine what organisms might be important in deterioration and industrial hygiene.

Undiluted heavy and emulsion type cutting oils in dilutions of 1–10 and 1–40 were employed. Organisms were inoculated into 5.0 ml. of sterile oil and checked by routine subculturing every twenty four hours. The survival times of thirty pathogenic and non-pathogenic organisms were determined.

Results show that Gram-negative bacteria are capable of surviving from 0 to longer than 250 days while Gram-positive organisms survived from 0 to 44 days. Some pathogens including enteric organisms were among those which survived for considerable time.

Bacteria are capable of surviving best in emulsion type cutting oils and these types of oil should have antibacterial agents incorporated into them. Organisms of choice in testing the efficiency of inhibitors in cutting oils would be *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Proteus vulgaris* and *Aerobacter aerogenes* since they are the most resistant of the thirty species tested.

Oxidation-reduction Potentials in Cultures of Clostridium Botulinum. Allen I. Laskin, and O. B. Williams, Department of Bacteriology, The University of Texas, Austin.

A technique has been developed for the measurement of oxidation-reduction potentials in cultures of *Clostridium botulinum*, in an attempt to investigate the relationship of this phenomenon and certain cell processes.

In media of low initial Eh values, strains of *Clostridium botulinum* exhibited rapid germination and growth, and lowered the potential of the medium to a characteristic range. Initiation of growth was slower in media which were more highly oxidized, but in those which did support growth, the final level of potential reached was in the same characteristic range.

Results so far show no relationship between any particular change in potential and the advent of spore formation in the cultures.

Preliminary Investigations of the Mineral Nutrition of Clostridium Botulinum. L. E. Bowers and O. B. Williams, Department of Bacteriology, The University of Texas, Austin.

Efforts were made to produce mineral deficiencies for *Clostridium botulinum* 62A in the medium of Roessler and Brewer and in the same medium less yeast nucleic acid and sodium thioglycollate. The omission of yeast nucleic acid and sodium thioglycollate increased the time required for the appearance of growth and tended to cause inconsistent results. The addition of ribose to the abbreviated medium tended to relieve the in-

consistencies and decrease the time required for the appearance of growth.

The omission of salts of iron, manganese, magnesium, and calcium, either individually or as a group, failed to produce any apparent mineral deficiencies as far as total growth is concerned, but under some conditions caused an increase in the time required for the appearance of growth.

When the two media were treated with 8-hydroxyquinoline according to the method of Waring and Werkman for the removal of trace elements, the results were essentially the same as those obtained with the untreated media.

It appears that the minerals firmly bound by the amino acids and glucose in the medium of Roessler and Brewer are sufficient for optimal growth of *Clostridium botulinum*.

In vitro Effects of Diamox on Sulfonamide and Antibiotic Activity. F. B. Engley, Jr., Rose Marie Steeg, and Arthur Ruskin, Departments of Bacteriology and Medicine, The University of Texas, Medical Branch, Galveston.

In vitro studies have been made with Diamox (acetazoleamide, Lederle) 2 acetyl amino 1, 3, 4 thiadiazole –5– sulfonamide. This compound is used clinically as a diuretic through its inhibiting action on carbonic anhydrase. These studies were designed to determine the effect of Diamox on the antibacterial action of representative sulfonamides, antibiotics and a furan derivative. Since this compound contains a sulfonamide radical, it was tested for antibacterial activity *per se* against *Stephylococcus aureus*, *Escherichia coli* and *Streptococcus viridans*. It was found that in its therapeutic range (50 micrograms per ml.) and at levels as high as 500 micrograms no antibacterial action was present against the test organisms. Tests were made by tube dilution tests to determine its effect on the anti-bacterial action of other drugs on the test organism. There was some suggestion that Gantrisin (Roche) and penicillin were slightly more active in the presence of high levels of Diamox against *Staph aureus* and *Strept viridans*. At therapeutic blood and urine levels the increased activity in the tube dilution tests was not significant. When tested by the paper disc method on agar high levels of Diamox appeared to inhibit some of the sulfonamides and several of the ten antibiotics under test. Of importance were the ob-

servations that Diamox did not interfere with the antibacterial action of the sulfonamides, antibiotics and furan derivative when tested at therapeutic levels.

AN UNKNOWN VIRUS-LIKE AGENT ISOLATED FROM A PATIENT AFFECTED WITH HODGKIN'S DISEASE*

REGINALD L. REAGAN, EDDY D. PALMER, EDWARD C. DELAHA, MILDRED T. STEWART AND A. L. BRUECKNER

Hodgkin's disease (Lymphadenoma) is a chronic advanced fatal disease of unknown etiology, manifested by painless and progressive enlargement of the lymph nodes and frequently of the spleen and liver. In the later stages, fever, anemia and cachexia are prominent. Many investigators consider it a malignant neoplastic entity; others an infectious granuloma. Various organisms have been suspected such as the tubercle bacillus, the Brucella organism and a filtrable virus. Hodgkin's disease is more prevalent among males than females, in the ratio of 7:3. It may occur at any age, but its greatest incidence is in the 3rd and 4th decades of life. The disease has little racial predilection (Merck Manual, 8th Ed.). The disease is a hyperplastic process which involves the reticulo-endothelial system and tends to become generalized. The lymph nodes, spleen, liver and bone marrow are chiefly involved.

Clinical Abstract

A 30-year old soldier, whose past medical history was not significant, was hospitalized because of two months of feverish feeling, night sweats and an enlarging neck. Examination showed low-grade fever and striking lymphadenopathy of the anterior node chains. The lacrimal gland on the left was moderately enlarged. The white blood count was 20,000, with 92% lymphocytes. The sedimentation rate was elevated. Chest plate showed bilateral hilar lymphadenopathy. The admission servical node biopsy revealed Hodgkin's disease.

Nitrogen mustard therapy, 37 mgm. over four days, produced almost complete resolution of the cervical nodes. A month later, however, both the cervical and the mediastinal nodes were larger, leading to radiation therapy, 2500 r (skin dose) to each cervical chain and 1500 r to the mediastinum. Response was good, with disappearance of toxic signs and symptoms. Blood was drawn

* From the Virus Laboratory, Live Stock Sanitary Service, University of Maryland, College Park, Maryland and the Gastrointestinal Section, Walter Reed Army Hospital, Washington, D.C. Thanks are due to Dr. Moulthrop and Dr. Porter of the University of Maryland Staff for testing this agent in chickens. Received for publication May 20, 1954.

for virus studies four and a half months following nitrogen mustard therapy and two months after the completion of radiation. The patient was at the time in remission.

Electron microscopic studies were conducted on erythrocytes from this patient and virus-like particles were noted on the surface of the erythrocyte which measured 120 mμ. in diameter (Reagan *et al.*, 1954).

Materials and Methods. Five cc. of venous blood were collected from the above patient and placed in sterile 50 cc. erlenmeyer flask which contained 7 mg. of heparin in 6 cc. of sterile physiological saline. The flasks of heparinized blood were placed in a —4° C. ice box for one hour. The flasks were then removed from the ice box and 20 embryonating eggs (6 to 8 days old) were inoculated in the allantoic cavity with 0.1 cc. of the heparinized blood. One embryo died on the 7th day post-inoculation, and the remaining eggs were discarded prior to hatching. The allantoic fluid and some of the yolk material were recovered from the dead embryo of the 1st culture and inoculated into 20 more embryonating eggs (6 to 8 days old) in the allantoic cavity. At the same time tests were conducted to determine the possibility of bacterial contamination. The same procedure of inoculating eggs, recovering allantoic fluid and yolk material from the dead embryos, pooling it, and inoculating 0.1 cc. of this material into 20 more embryonating eggs (6 to 8 days old), and sterility cultures were continued throughout the experiment to the 10th passage. These passages are being continued at the present time. Allantoic fluid and yolk material from the 8th subculture were inoculated into 3 groups of mice and hamsters intracerebrally, intraperitoneally, and intranasally to see if an agent pathogenic for these animals was present.

Neutralization tests were conducted on the allantoic fluid from the 10th subculture to determine if the agent could be either the Newcastle disease virus or bronchitis virus, sometimes present in embryonating eggs.

Results and Discussion

It was observed that of the embryonating eggs inoculated an increasing number died in each succeeding subculture as shown in Table 1. In the 8th subculture 50 per cent of the embryos died whereas only 5 per cent of the embryos died in the 1st culture. The sterility tests which were conducted on each subculture

were negative and thus eliminated the possibility of bacterial contamination. All of the mice and all of the hamsters in the three groups which were inoculated intracerebrally, intranasally, and intraperitoneally appeared normal at the end of a 21 day observation period. This eliminated the possibility of the presence of a neurotropic or other agent. The neutralization tests were negative for the two viruses stated above. The 10th embryo passage material was injected into 6 six-week-old chickens by intramuscular and intratracheal inoculation. The chickens showed no clinical signs of a respiratory disease.

TABLE 1

Response through 10 passages of 6–8 day embryos to inoculation in the allantoic cavity of an unknown agent isolated from a patient affected with Hodgkin's Disease

Passage	No. eggs inoculated	Age of embryo in days	No. embryos dead	Minimum to Maximum* incubation period in days
1	20	6–8	1	7
2	20	6–8	3	6–8
3	20	6–8	3	6–8
4	20	6–8	4	6–8
5	20	6–8	6	6–8
6	20	6–8	7	6–8
7	20	6–8	8	6–8
8	20	6–8	10	6–8
9	20	6–8	10	6–8
10	20	6–8	10	6–8

Blood was collected from a patient affected with Hodgkin's disease and injected into the allantoic cavity of 20 (6 to 8 day old) embryonating eggs. One egg died and the allantoic fluid was collected and re-injected into 20 more embryonating eggs continuing through the 10th passage. Further passages are being continued at the present time. Table 1 shows that while only 5 per cent of the embryos died in the first subculture, the mortality increased progressively and at the 10th subculture 50 per cent of the embryos died. Intracerebral, intraperitoneal, and intranasal inoculation of mice and hamsters showed that no agent with neurotropic or other properties was present. Neutralization tests indicate that the agent is not Newcastle disease virus or bronchitis virus which might have been dormant in the eggs. The chickens showed no signs of illness after exposure to this agent

intramuscularly and intratracheally. It is assumed that this agent was isolated from the blood of a patient affected with Hodgkin's disease. However, the possibility that this agent is some virus which is not necessarily connected with Hodgkin's disease can not be definitely excluded.

Electron Microscopic Studies

Thirty cc. of allantoic fluid from the 10th egg embryo passage were used. This fluid was collected using sterile technique from embryonating eggs of the 10th passage, infected with a virus-like body isolated from a patient's blood affected with Hodgkin's disease (which was described in a previous paper, Reagan *et al.*) and placed in a sterile flask. Seven-day old embryonating eggs were used for the inoculation. Death of the infected embryos occurred between the 6th and 8th day post-inoculation. Thirty cc. of normal allantoic fluid were also obtained from embryonating eggs of the same age using sterile technique and put into a sterile flask.

These two suspensions were then subjected to 5 minutes centrifugation at 1,000 rpm. in an angle centrifuge. The supernatants were removed from the two specimens and filtered through a Type St Size L3 Seitz filter. The filtrates from the two suspensions were then subjected to centrifugation under refrigeration in a Sinco ultracentrifuge for two hours at 44,000 rpm. The temperature of the refrigerated outer jacket stayed constant at —14° during the centrifuge run. After the 2 hour centrifugation period, the supernatants from the specimens were discarded, and the sediment from each specimen was resuspended in 1.0 cc. of distilled H_2O.

This filtered material was inoculated into 7-day old embryonating eggs. A 50 per cent mortality rate was observed with death of the embryos occurring 5 and 7 days post inoculation.

A small drop of each suspension was placed on several parlodion film supports. After all excess fluid had been removed with small capillary pipettes, the films were dried and shadowed with chromium (Wiliams, 1946) at arc tangent 1/6 and examined under the R.C.A. electron microscope, type E.M.U.

Electron microscopic examination of the concentrated virus suspension revealed virus-like particles as shown in Figures I

and II. These particles were small and spherical, ranging in diameter from 100 mμ–110 mμ and resembled the virus-like particles found in the erythrocytes of the same patient as described in a previous paper by Reagan *et al.* Upon examination of the

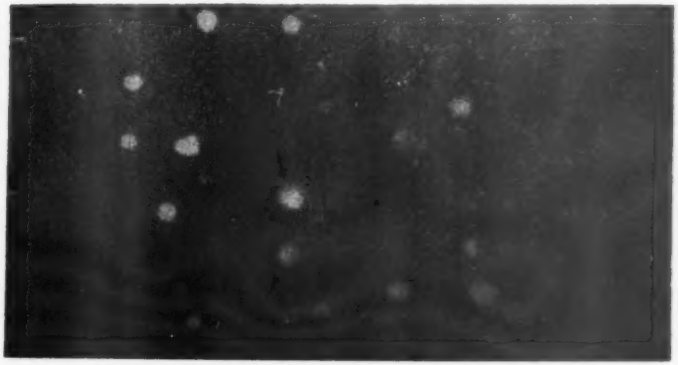


FIG. I. Electron micrographs of an agent isolated from a patient affected with Hodgkin's Disease. Shadowed with chromium at arc tangent 1/6. x 64000.

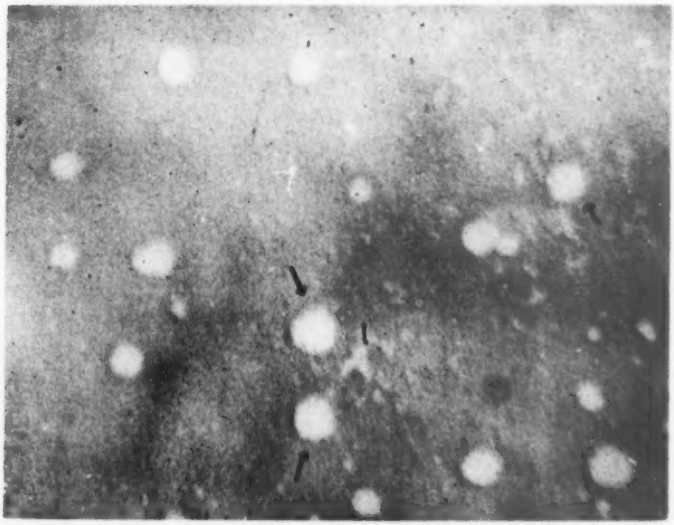


FIG. II. Electron micrographs of an agent isolated from a patient affected with Hodgkin's Disease. Shadowed with chromium at arc tangent 1/6, x 82000.

concentrated suspensions of the normal allantoic fluid no such virus-like particles were seen.

Summary

Studies by electron microscopy of the allantoic fluid from embryonating eggs, infected with material isolated from the blood of a patient affected with Hodgkin's disease, showed small spherically shaped virus-like particles from 100 mμ to 110 mμ in diameter. No such particles could be demonstrated in normal allantoic fluid.

These bodies confirm to morphology as described previously.

REFERENCES

Reagan, R. L., E. Palmer, E. Delaha, M. T. Stewart, and A. L. Brueckner: Electron Microscopic Studies of Erythrocytes from a Patient Affected with Hodgkin's Disease. *Tex. Rep. on Biol. and Med.*, 12: 178–181, 1954.

Williams, R. C., and R. W. G. Wyckoff: Application of Metallic Shadow-casting to Microscopy. *Jour. Applied Physics*, 17–23, 1946.

ELECTRON MICROSCOPIC STUDIES OF AN EGG-ADAPTED VIRUS ISOLATED FROM A PATIENT WITH A COMMON COLD*

REGINALD L. REAGAN, EDDY D. PALMER,† EDWARD C. DELAHA, SUE R. COOK, ARTHUR L. BRUECKNER, AND HELEN E. NELSON

The "common cold" (acute coryza, acute rhinitis) is attributable to infection of the upper respiratory tract with a virus. The symptoms and signs indicate the presence of an acute but transient alteration in the physiology of the mucous membrane of the upper respiratory tract, particularly that lining the nose and the paranasal sinuses (Horsfall, 1952). All of the etiologic factors are not yet completely understood, but there is good evidence indicating that one or more viruses may be involved in a "cold."

Kruse (1914) and Foster (1916) successfully transmitted colds experimentally to man by means of filtered materials. More than 10 reports have confirmed their findings while 5 others have failed to confirm them. Dochez *et al.* (1930), first reported the successful transmission of the common cold to chimpanzees by means of filtrates. Dochez *et al.* (1931), reported the cultivation of the virus in tissue-culture medium and Kneeland *et al.* (1936), described the cultivation of the agent on the chorio-allantoic membrane of the chick embryo. Pollard *et al.* (1947), Topping *et al.* (1947), and Ward *et al.* (1950), described cultivation of the virus in the allantoic sac of embryonating chicken eggs. Andrewes *et al.* (1949, 1950), have been unsuccessful in attempts to cultivate their strains in the chick embryo. All mammalian species tested, except man and chimpanzees, appear to be insusceptible to infection with the etiologic agent (Andrewes, 1949). Colds, experimentally induced by the intranasal inoculation of bacteriologically sterile filtrates

* From the Virus Department, Live Stock Sanitary Service Laboratories, University of Maryland, College Park, Md., and the Gastrointestinal Section, Walter Reed Army Hospital, Washington, D.C. Received for publication August 9, 1954.

† Chief Gastrointestinal Section, Walter Reed Army Hospital, Lieutenant Colonel, Medical Corps, U. S. Army and Consultant in Gastroenterology to the Surgeon General.

or chick-embryo-passage material, closely simulate natural colds and show all the features of the naturally acquired disease.

Recent studies by Andrewes (1949) with collodion membranes of graded porosity indicate that the virus may have dimensions of 40–50 mμ or less. Andrewes' (1950) results of tests for immunity to reinfection suggest that resistance may develop and persist for three or four months.

In a previous study (Reagan *et al.*, 1954) by electron microscopy of filtered infected nasal and throat washing from an adult affected with a common cold, spherical virus-like particles with a diameter of 80–90 mμ were observed. This virus was capable of inducing a cold in one chimpanzee. These virus-like bodies were not observed in normal nose and throat washings.

Materials and Methods

Nasal and throat saline washings were obtained two days after the onset of a common cold from a physician at the Walter Reed Army Hospital. The clinical picture was as follows: This 34 year old physician had had simple colds approximately twice a year for many years. He had no known allergies. One evening he recognized the prodromals of one of his usual colds. His head felt full and he had a slight headache. In the morning he had the typical symptoms of a common cold. Examination showed a temperature of 100° F., mild conjunctival infection, pharyngeal redness and mild edema, and nasal congestion. Nasal smears showed no eosinophils. Nasal and pharyngeal washings were taken for virus studies 14 hours after onset. The cold ran a course of 6 days and was not complicated by significant secondary infection.

The infected nasal and throat washings from the ill physician was filtered through a Type St Size L3 Seitz filter. This filtrate was then used as the inoculum to initiate the experiment.

Twenty 7-day-old embryonating chicken eggs were each inoculated in the chorio-allantoic cavity with 0.2 ml. of the nasal and throat washings filtrate. Six to eight days post inoculation 15 per cent of the embryos were dead. Upon death of the embryos, the allantoic fluid was aseptically removed from each egg, pooled, and used as the inoculum for 20 more 7-day-old embryonating chicken eggs. This procedure of transferring the chorio-allantoic fluid from dead eggs to more embryonated eggs was repeated for 20 passages. The mortality rate never rose above 20 per cent and the incubation period ranged from 5 to 10 days throughout the 20 passages.

The chorio-allantoic fluid from the dead eggs of the 20th passage was aseptically removed, pooled and filtered through a Type St Size L3 Seitz filter. Chorio-allantoic fluid was also harvested from 6 normal embryonated chicken eggs and was similarly pooled and filtered through a Type St Size L3 Seitz filter. The 2 filtrates were then subjected to centrifugation under refrigeration in a Spinco ultracentrifuge for 2 hours at 44,000 R.P.M. The temperature of

the refrigerated outer jacket stayed constant at —8° C. during the centrifugation. After the 2 hour centrifugation the supernatants from the 2 specimens were discarded, and the sediments from each specimen were resuspended in 1.0 cc. of chilled physiological saline. Several drops of each specimen were placed on several parlodion film supports which had been prepared 92 hours previously. After all excess fluid had been removed with small capillary pipettes the films were dried and shadowed with chromium (Williams and Wyckoff, 1946) at arc tangent 2/12 and examined under the R.C.A. electron microscope, type E.M.U. The balance of the infected material was nasally instilled into 2 human volunteers who prior to exposure were completely normal. They were observed daily by one of the co-authors.

Results

Table 1 demonstrates the mortality rate and the incubation period during the 20 egg passages. It is observed that the mortality rate ranged between 5–20 per cent and that it never rose

TABLE 1

The response of embryonated chicken eggs to a virus isolated from a patient with a common cold

Passage No.	No. of Eggs Inoculated	No. of Eggs Dying	Mortality Rate Per Cent	Incubation Period (days)
1	20	3	15	6–8
2	20	2	10	6–9
3	20	3	15	6–8
4	20	2	10	5–7
5	20	4	20	6–8
6	20	3	15	6–9
7	20	4	20	6–9
8	20	2	10	6–9
9	20	2	10	6–8
10	20	2	10	6–9
11	20	1	5	5–10
12	20	4	20	6–8
13	20	4	20	5–7
14	20	3	15	6
15	20	4	20	5–8
16	20	4	20	6–8
17	20	2	10	7–8
18	20	2	10	7–8
19	20	4	20	6–8
20	20	4	20	6–10

above the 20 per cent figure, even in the later passages. The period of incubation averaged about 7 days throughout.

Within 24 hours after nasal instillation of the infective chorioallantoic fluid, both of the human volunteers evidenced mild,

but typical symptoms of a common cold: sneezing (both patients), nasal and pharyngeal congestion (one patient), scratchy throat (both patients) and loss of energy (both patients). Forty-eight hours after exposure, both volunteers evidenced more typical symptoms of having common colds: nasal and pharyngeal elaboration of mucous and mild coughing.

Figures I, II, and III are electron micrographs of the infective

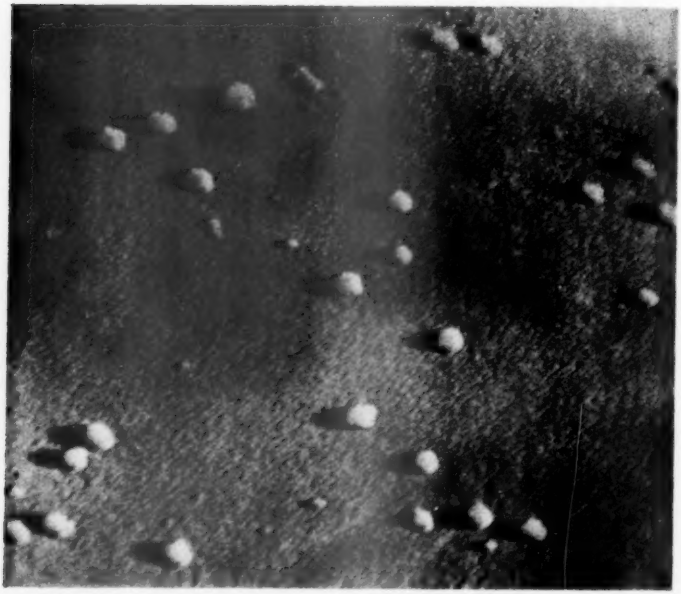


FIG. I. Electron micrograph of a strain of the common cold virus after passage in embryonated chicken eggs. Shadowed with chromium at arc tangent 2/12, x 40,000.

chlorio-allantoic fluid from the 20th embryonated egg passage. The micrographs reveal the presence of small, spherical, virus-like bodies which have a diameter of 95–100 mμ by direct measurement. These bodies are similar to those observed earlier by Reagan *et al.* (1954), in the nasal and throat washings from a patient with a common cold. The bodies could not be demonstrated in the chorio-allantoic fluid filtrate from normal embryo-

nated chicken eggs which had been subjected to the same procedure of concentration and electron microscopic examination.

Summary

Nasal and throat saline washings were obtained from a patient 2 days after the onset of a common cold and was filtered through a Type St Size L3 Seitz filter. This filtrate was used to inoculate 20 7-day-old embryonating chicken eggs in the chorio-allantoic cavity. Six to 8 days post inoculation 15 per cent of the embryos

FIG. II. Electron micrograph of a strain of the common cold virus after passage in embryonated chicken eggs. Shadowed with chromium at arc tangent 2/12, x 72,000.

were dead. Upon death the chorio-allantoic fluid was asceptically removed, pooled, and used as the inoculum for 20 more 7-day-old embryonating chicken eggs. This procedure was carried out for 20 egg passages and upon death of 20 per cent of the embryos of the 20th passage the choria-allantoic fluid was aseptically removed, pooled, and filtered. Chorio-allantoic fluid was also har-

vested from normal embryonated chicken eggs and filtered. The 2 filtrates were then centrifuged and prepared for electron microscopic examination. The balance of the infected material was nasally instilled into 2 human volunteers who developed mild, but typical symptoms of a common cold.

Electron micrographs of the infective chorio-allantoic fluid from the 20th embryonated egg passage reveal the presence of

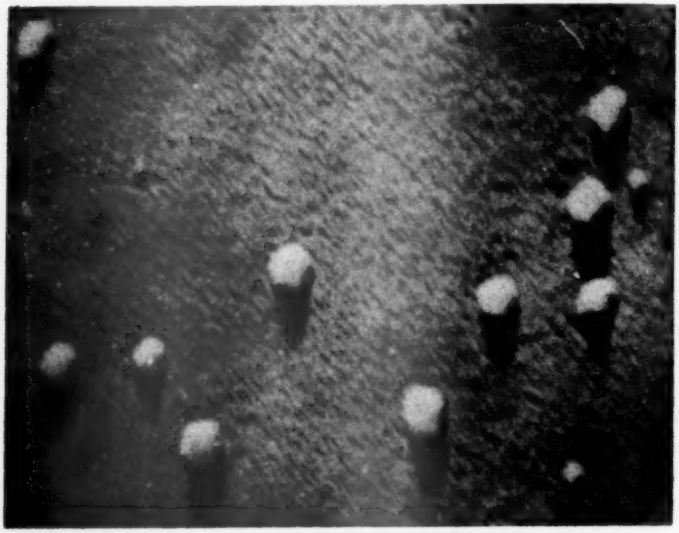


FIG. III. Electron micrograph of a strain of the common cold virus after passage in embryonated chicken eggs. Shadowed with chromium at arc tangent 2/12, x 108,000.

small, spherical, virus-like bodies which have a diameter of 95–100 mμ by direct measurement. These bodies are similar to those observed by Reagan *et al.* (1954), in the nasal and throat washings from a patient with a common cold. The bodies could not be demonstrated in the chorio-allantoic fluid filtrate from normal embryonated chicken eggs which had been subjected to the same procedure of concentration and electron microscopic examination.

REFERENCES

Horsfall, F. J.: *Viral and Rickettsial Infections of Man.* Thomas M. Rivers, Second Edition, J. B. Lippincott Co., Philadelphia, 1952.

Kruse, W.: Die Erreger von Husten and Schnupfen. *Munch. Med. Wchnechr.*, 61: 1547, 1914.

Foster, G. B., Jr.: The Etiology of Common Colds. The Probable Role of a Filterable Virus as the Causative Factor: A Preliminary Note. *J. Am. Med. Assn.*, 66: 1180–1183, 1916.

Dochez, A. R., G. S. Shibley, and K. C. Mills: Studies in the Common Cold. IV. Experimental Transmission of the Common Cold to Anthropoid Apes and Human Beings by Means of a Filterable Agent. *J. Exp. Med.*, 52, 701–716, 1930.

Dochez, A. R., K. C. Mills, and Y. Kneeland, Jr.: Study of the Virus of the Common Cold and Its Cultivation in Tissue Medium. *Proc. Soc. Exp. Biol. and Med.*, 28: 513–516, 1931.

Kneeland, Y., Jr., K. C. Mills, and A. R. Dochez: Cultivation of the Virus of the Common Cold in the Choric-allantoic Membrane of the Chick Embryo. *Proc. Soc. Exp. Biol. and Med.*, 35: 213–215, 1936.

Pollard, M., and C. D. Caplovitz: Experimental Studies With the Agent of the Common Cold. *Science*, 106: 243–244, 1947.

Topping, N. H., and L. T. Atlas: The Common Cold: A Note Regarding Isolation of an Agent. *Science*, 106: 636–637, 1947.

Ward, T. G., and D. F. Proctor: Isolation of a Common Cold Virus in Chick Embryos and the Clinical Manifestations It Produces in Human Volunteers. *Am. J. Hyg.*, 52: 91–106, 1950.

Andrewes, C. H.: The Natural History of the Common Cold. *Lancet*, 1: 71–75, 1949.

Andrewes, C. H.: Adventures Among Viruses. III. The Puzzle of the Common Cold. *New England J. Med.*, 242: 235–240, 1950.

Reagan, R. L., E. D. Palmer, M. T. Stewart, and A. L. Brueckner: Electron Microscopic Studies of a Virus Isolated from a Patient with a Common Cold. *Tex. Rep. Biol. and Med.*, 12: 174–177, 1954.

Williams, R. C., and R. W. G. Wyckoff: Application of Metallic Shadow-casting to Microscopy. *J. Applied Physics*, 17: 23–26, 1946.

A SEPARABLE AND MULTIPURPOSE TISSUE CULTURE CHAMBER*

GEORGE ROSE

The evolution of the tissue culture chamber has followed the basic pattern of most scientific developments in that one idea has planted seeds in the minds of others only to there mutate and give birth to refined variants. This series of mutations can easily be followed through the literature which contains many variations of the original modes, techniques and devices. The coverslip method and the tube and flask systems stand as the two basic tools of culturing from which the now many methods have germinated.

Carrel (1923) developed special optically thin flasks which have endured the test of time. Maximow (1929) introduced the double coverslip variation of the hanging drop as a method of renourishing cells for extended cultivation. Roller tubes have been flattened by Porter *et al.* (1945), and ring slides and perforated steel slides have been used by Gey (1950), each designed for optical improvement. Pomerat (1951) uses hanging drop or roller tube coverslips and applies them to an all-glass and paraffin-wax system for perfusion during cinematographic recording. More recently, Buchsbaum and Kuntz (1953) have perfected a masterful apparatus of controlled perfusion through a double coverslip chamber mounted in a stainless steel slide. A similar system using a plastic or glass slide and double coverslips has been demonstrated by Christiansen *et al.* (1953).

The chamber presented here (Figs. 1, 2, 3, and 4) is still another variation which attempts to approach the ideal conditions for cultivation, optical observation and nutrient control. Unlike its predecessors which are generally of all-glass construction so far as the actual vault is concerned, this chamber employs pure gum latex which allows for the unique use of syringe techniques throughout the entire cultivation period. The chamber may be entered innumerable times without septic

* From the Tissue Culture Laboratory, Arabia Temple Crippled Children's Clinic, Hermann Hospital, Houston. Supported by a grant-in-aid from the American Cancer Society administered by C. M. Pomerat. Received for publication August 10, 1954.

FIGS. 1 to 4. Photographs of the chamber described in the text showing its component parts and adaptability as a tissue culture method.

FIG. 1. The component parts of one chamber.

FIG. 2. The chamber adapted to a perfusion system.

FIG. 3. A chamber on the microscope stage ready for phase contrast observation. The countersunk surface is up for the accommodation of the high power objectives when viewing peripheral cells.

FIG. 4. The ease of exchanging fluids aseptically without the usual culture room prophylactic ritual.

sequelae. The latex is without apparent toxic effect although a systematic study of its merits has not yet been attempted.

In developing this device the following points were considered: a chamber that (1) after its usefulness for a particular culture could be dismantled and the cells fixed, stained and slide mounted for a permanent record, (2) would permit microscopic observation of oil magnitude at any desired time without altering the chamber or the environment of the cells, (3) could be perfused or its nutrient exchanged at will, (4) possessed good aseptic technical qualities, (5) contained adequate surface for extended culturing, (6) used relatively small amounts of nutrient, (7) could dispense with plasma clots, (8) would permit vital staining without disrupting the cellular growth of altering the structure of the chamber, and finally (9) presented a system of cultivation conducive to culture survival over long periods of time. The construction of the chamber described below achieves the fulfillment of these desired features.

Materials and Methods

Description of Parts. This chamber is actually a bolted sandwich composed of five layers. There are two stainless steel plates, one rubber gasket and two coverslips. These five parts are laminated in a methodical manner and secured by four Allen headed bolts.

Steel Plates. The retaining plates are stainless steel and measure 2 x 3 x ⅛ inches. Each plate has a center hole which is 1 1/16 inches in diameter and beveled at a 45 degree angle. The hole of the bottom plate has a 1/16 inch countersink which extends ⅛ inch from the perimeter. This produces an area for the objectives of high magnification when they approach the edge of the chamber (Fig. 3). Each plate has four corner holes; those of the top plate being countersunk to receive the flat heads of the Allen bolts and those of the bottom plate being tapped to receive their threads.

Bolts. These are standard flat Allen headed bolts No. 10 x 32 x ⅜ (.190 inch diameter x 32 threads per inch x ⅜ inch overall length). As the thickness of the assembled chamber is only slightly greater than ⅜ inch these bolts allow for flush surfaces.

Rubber Gasket. This central component of the chamber is made from pure gum latex (floating stock) and measures 2 x 1¾ x ⅛ inches and has a center hole which is 1 3/32 inches. As the rubber is slightly compressed when the chamber is formed this hole closely approaches that of the steel retaining plate's so that the pressures on the glass parts remain relatively constant.

Coverslips. These parts are standard optical coverslips of No. 1 thickness and measure 50 x 43 mm. which is approximately the dimensions of the rubber gasket.

Assembly and Culturing. There are two methods which may be followed according to the size and type of tissue to be cultivated. If small fragments are explanted the closed method may be used most advantageously but if large fragments are used or plasma clots desired the open method must be used. Following cleansing of the glassware and rubber by standard methods the coverslips are dry heat sterilized and the rubber gaskets autoclaved in Petri dishes with pieces of hard paper between them to prevent cohesion.

Closed Method. By this method the chamber is first assembled and the fragments afterwards injected into the prefabrication. The so-formed chamber may be stored until ready for cultivation procedures. The bottom plate is placed in front of the technician with the beveled side down. Using a 100 mm. Petri dish cover for dust protection a sterile coverslip is flamed and placed on the center of the retaining plate with the 50 mm. edge parallel to the 3 inch edge of the plate. A rubber gasket followed by another coverslip is then placed over the assembly. The glass and rubber edges are aligned with two forceps and the top retaining plate then positioned. The four bolts are dropped into their holes and slowly tightened with the Allen wrench. Final tightening is done only after a No. 25 half-inch needle has been inserted into the chamber through the edge of the gasket which permits the pressure within the vault to equalize with the local atmosphere. Failure to observe this precaution may result in a coverslip blowout as the bolts are tightened and the internal pressure rises. This tightening phase may at first appear unreliable but after a few practice attempts the desired skill becomes automatic. The needle is removed and the chamber set aside until needed.

Of particular interest while developing the closed method has been routine studies on technique using a stable strain of human

malignant epithelial cells (strain HeLa) which was derived from an epidermoid carcinoma over three years ago by Gey *et al.* (1952). HeLa cells growing on the glass walls of roller tubes are washed in Earle's balanced salt solution (BSS) and then removed with a rubber policeman by simply scraping the cultures into the fluid. After flaming the lip of the tube it is practical and bacteriologically safe merely to pour its contents into a sterile Petri dish. The cultures are then transferred with the aid of a spatula to a glass plate and cut with two scalpels into fragments ranging from 0.1–0.5 mm. in diameter. A 2 cc. syringe fitted with a No. 20 half-inch needle and filled with a nutrient fluid is used to pick up a desired number of fragments by placing a few drops on the cutting plate and aspirating this with the fragment(s) into the syringe. The nutrient used in these technique experiments was composed of 6 parts of a 50–50 mixture of non-malignant ascitic and pleural fluids and 5 parts of Earle's BSS. The cells and fluid are next injected through the gasket and into the chamber with the aid of a second needle (No. 25 half-inch) which acts as an air vent. After the chamber is filled (approximately 1.8 cc.) the needles are removed and the fragments adjusted toward the center on the lower coverslip which is adjacent to the countersunk retaining plate. As the HeLa cell fragments are denser than the nutrient this adjustment is simply a tilting procedure. After one day of incubation the HeLa cells are firmly adherent to the glass coverslips and epithelialization is evident. The culture fluids are generally changed weekly although occasionally when there are several explants in one chamber semiweekly changes are necessary. The rule has been to change the supernatant fluids as the phenol red (.001 per cent) indicates a shift to acid. Usually the cultures are washed in BSS for 5 minutes at the time of nutrient change. The double needle single syringe technique must be reiterated as the results are far superior to attempts with a single needle and syringe. The danger of the single needle method is twofold; one being the likelihood of coverslip breakage and the other being the promiscuity of air bubbles that are prone to occur. The two needles are most effective if they are inserted on the same side about a quarter of an inch apart (Fig. 4). A cotton plug in the vent needle serves as a convenient bacteriological filter. Because this chamber is only ⅛ inch in depth, air bubbles of major proportions as ¼ cc. and

larger are apt to lodge directly over the cells and allow drying. For this reason the amount of fluid used in each chamber should closely approach its capacity of 1.8 cc. It is felt, however, that a small bubble is advisable to act as a buffer toward changes in temperature as exist between air-conditioned culture rooms and the incubator.

By following these procedures HeLa cells have been maintained in continuous culture for 58 days (at the time of writing) and have had an average of one needle per day inserted and removed from its chamber without resultant contamination. Actually six needles are used in one day as required for removal of supernatant, replacement with the BSS wash, removal of wash and final replacement with fresh supernatant nutrient. Cultures have been viewed daily with long working distance phase contrast 21x and 97x objectives (B&L) without any obvious light injury from this repeated assault (Figs. 5 and 6). One HeLa culture, was started from a tiny spherical morula-like cluster approximately 0.02 mm. in diameter. After 34 days of cultivation these cells had epithelialized and produced a sheet of single-layered cells which measured 5.7 x 4.8 mm. After this time presumably due to internal stresses of contraction the culture became lacy but nonetheless active. Although the diameter was only slightly increased after 45 days of cultivation the cells continued to proliferate and fill in the lacy areas. The central syncytium remained intact but peripherally the cells were often spindles or large epithelioid giant cells. Mitosis has been a constant feature of this culture. At 53 days when the fluids were exchanged the supernatant had an additional component of 5 per cent embryo extract ultrafiltrate (50–50 Chick-Beef). At 55 days the cells appeared larger, more epithelioid and mobile. The intercellular spaces seemed to have spread and the periphery of the culture had a diminished number of spindle forms. The greatest diameters at this time were 6.3 x 6.5 mm. which appear to be attributed to the two-day influence of the growth stimulating embryonic extract. Another chamber which contained three fragments produced a sheet of cells which measured 15 x 10 mm. in 14 days. At this time the culture was fixed for staining (Fig. 7).

Open Method. If the specimen is too large to pass through the bore of a No. 20 needle or if a plasma or cellophane substrate is

desirable this method must be used. The explant(s) is placed in the chamber before the top coverslip is positioned and the remaining procedures after closing remain the same.

Separation and Fixation. When cultures are to be fixed, stained and mounted the following procedure using the double-needle technique has been found most satisfactory:

(1) Remove the supernatant; (2) Replace with warm BSS for 30 minutes; (3) Remove wash solution and inject fixative; (4) After fixation leave one needle inserted in the gasket and remove the Allen bolts; (5) Lift off the retaining plates and the coverslips and gasket will be found to be adherent; (6) Immerse these fused parts in the fixative (water if applicable) for several minutes; (7) Apply gentle pressure with the syringe barrel and one coverslip will separate partially. This must then be lifted off very gently; (8) Lift the gasket off of the other coverslip; (9) Stain the culture coverslip.

As these coverslips are an unusual size for the conventional staining jars, a suggested arrangement may be devised using museum jars (50 x 50 x 30 mm.). These are more conveniently accessible if they are snugly fitted in a specially constructed wooden box.

Discussion

This chamber is somewhat unique in that it permits a multiplicity of functions which are not found in any other standard method. Although this chamber was not designed to replace accepted methods commonly used, it is felt that as a supplement it will prove valuable. There are certain obvious disadvantages to such a system as well as an apparent overbalancing number of advantages. The initial cost of stainless steel instruments is high but they are virtually indestructible so that the maintenance cost becomes that of only the coverslips expended. The

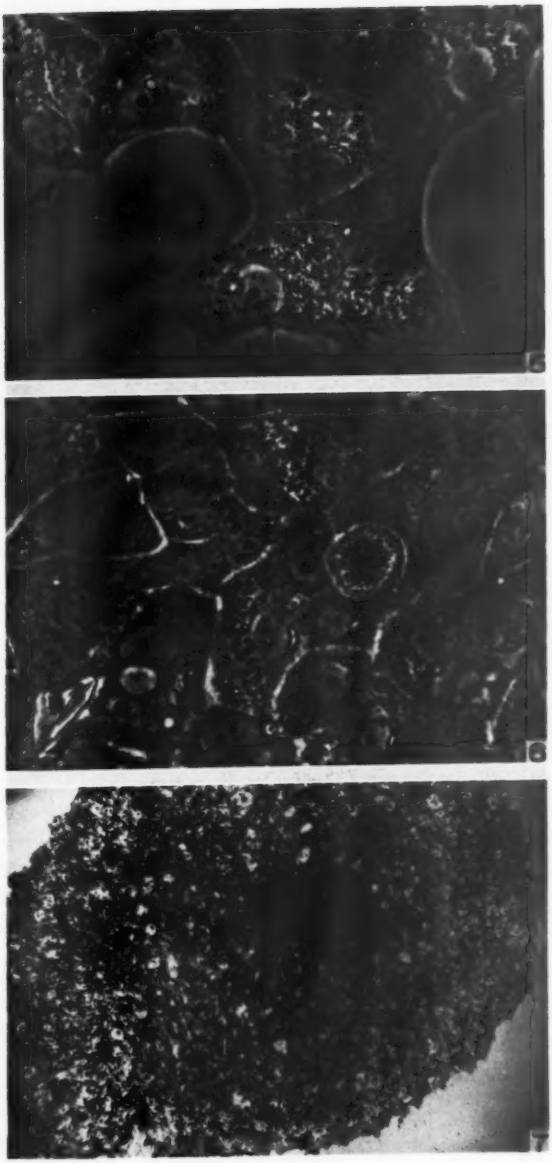
---

FIGS. 5 to 7. Photomicrographs of strain HeLa cells cultivated in the chamber under phase contrast and fixed conditions. The cells are growing directly on the glass.

FIG. 5. Low power phase contrast 21x objective and 10x projection ocular (B & L). 5 day culture.

FIG. 6. Same as figure 5.

FIG. 7. Very low power photograph using 48 mm. objective without ocular. Area represent approximately 5 mm. segment of 14-day HeLa culture fixed with formalin and stained with hematoxylin.



small size of the chamber permits quicker shifts in pH values. This can be interpreted as being a disadvantage in that a more vigilant observance of the cultures is required and that the fluid nutrients must be changed more frequently or removed and their pH adjusted to fall within a compatible range. This may also be interpreted as being of potential diagnostic value in that pH shifts are more readily detected because of this lack of space for gas diffusion. A sequel to this is a projected study involving the difference in pH shifts of culture fluids composed of malignant and "normal" sera in tissue culture. HeLa cells have always acidified the nutrient fluid in these chambers as well as in roller tubes with the same nutrient. The rate or degree of shift may render a constant comparison between these two types of sera. Another projected use of this chamber worth mentioning at this time is its potential use in the study of nuclear rotation over prolonged periods of time (Pomerat, 1953). This living feature of cells may yield an analytical method which might be tagged to cells cultivated under the aegis of malignant serum as compared to "normal" serum. Of considerable importance is the ability of cells in this chamber to be observed under phase contrast optics of all powers (10X–97X) without any special preparation. The fact that a perfusion system can be plugged into this chamber by the mere insertion of a few needles which are attached to tubes and supply bottles distinguishes this chamber as something different in the tissue culture field. Vital staining is equally as easily carried out. The size of the rubber gaskets were arrived at by trial and error. Thinner ones would permit only small bore needles and increase the chance of glass breakage. Thicker gaskets would require more media and produce poorer conditions for phase contrast observation. Although only No. 20 needles are the largest recommended, occasionally No. 18 may be used with extreme delicacy for larger tissue fragments when using the closed method. A recent innovation I will merely mention is the feasibility of subculturing from chamber to chamber.

Appreciation is expressed to the Wahlberg-McCreary Company of Houston for their cooperation during the development phase and for their excellent craftsmanship in turning out the finished product.

REFERENCES

Buchsbaum, R., and J. Kuntz: The Effects of Certain Stimulants and Depressants on Individual Fibroblasts in a Perfusion Chamber. *Proc. New York Acad. Sciences* (manuscript in press), 1954.

Carrel, A.: A Method for Physiological Study of Tissues *in vitro*. *J. Exper. Med.*, 38: 407–418, 1923.

Christiansen, G. S., B. Danes, and L. Allen: A Culture Chamber for the Continuous Biochemical and Morphological Study of Living Cells in Tissue Culture. *Exptl. Cell Research*, 5: 1015, 1953.

Gey, G. O.: Personal communication in Parker's *Methods of Tissue Culture*. (2d ed., New York, Paul B. Hoeber, Inc., 1950).

Gey, G. O., W. D. Coffman, and M. T. Kubicek: Tissue Culture Studies of the Proliferative Capacity of Cervical and Normal Epithelium. *Cancer Research*, 12: 264–265, 1952.

Maximow, A.: Ueber die Entwicklung Argyrophiler und Kollagener Fasern in Kulturen von Erwachsenem Saugetiergewebe. *Ztsch. f. Mikr. Anat. Forsch.*, 17: 625, 1929.

Porter, K. R., A. Claude, and E. F. Fullam: A Study of Tissue Culture Cells by Electron Microscopy. Methods and Preliminary Observations. *J. Exper. Med.*, 81: 233–245, 1945.

Pomerat, C. M.: *Methods of Medical Research* (Vol. 4, Year Book Publishers, Chicago, 198–291, 1951).

Pomerat, C. M.: Rotating Nuclei in Tissue Cultures of Adult Human Nasal Mucosa. *Exptl. Cell Research*, 5: 191–196, 1953.

CALLING ATTENTION TO

Items of Possible Interest to Our Friends

July, 1954

Clinical: RH Barnes & Co. find group psychotherapy helpful in emotional problems of multiple sclerosis (*Group Psychoth* 6/193/54) . . . K Boman & E Jalavisto note marked impairment of acuity of weight discrimination in old persons (*Acta Physiol Scand* 30/303/54) . . . FW Clements gives etiology of knock-knees (*Med J Austral* 1/737/May 15/54) . . . RF Escamilla offers *Laboratory Aids in Endocrine Diagnosis* (CC Thomas, Springfield, Ill, 54, 131pp, $3) . . . J Fine discusses *The Bacterial Factor in Traumatic Shock* (CC Thomas, Springfield, Ill, 54, 82pp, $2.5) . . . GH Fletcher & Co. discuss use of cobalt 60 in interstitial & intracavitary radiation therapy (*Am J Roentg* 71/1021/54) . . . R Griffith reports *The Abilities of Babies* (McGraw-Hill, NY, 54, 239pp, $6.5) . . . A Grollman describes *Acute Renal Failure* (CC Thomas, Springfield, Ill, 54, 92pp, $3) . . . PD Hart edits symposium on tuberculosis (*Brit Med Bull* 10/73–155/54) . . . PC Hodges gives Caldwell Lecture on normal, diseased & dead bone (*AM J Roentg* 71/925/54) . . . DM Little & CR Stephen propose balanced anesthesia (*Anesth* 15/246/54 no references) . . . MC Longerich & J Bordeaux develop *Aphasia Therapeutics* (Macmillan, NY, 54, 185pp, $3.75). . . FDW Lukens discusses *Medical Use of Cortisone* (Blakiston, NY, 54, 534pp, $8) . . . Jar Miles discusses benign lymphatic meningitis with reference to mumps as cause (*Med J Austral* 1/659/May 1/54) . . . JW Noah & A Branc find ragweed pollen releases histamine from red blood cells (*J. Allergy* 25/210/54) . . . EJ Pulaski tells how to control *Surgical Infections* (CC Thomas, Springfield, Ill., 54, 332pp, $7) . . . For us old backers, GK Rose discusses backache & that disc (*Lancet* /1143/June 5/54) . . . J Roskam discusses *Arrest of Bleeding* (CC Thomas, Springfield, Ill, 54, 81pp, $2.5) . . . K Schwarz edits symposium on nutritional factors & liver disease (*Ann NY Acad Sci* 57/615–962/54) . . . MJH Smith & Co show salicylate action is independent of pituitary-adrenal system (*Lancet* 266/1008, 1010/May 15/54) . . . I Skottowe outlines *Clinical Psychiatry* (McGraw-Hill, NY, 54, 395pp, $8.75) . . .

Pre-clinical: PO Astrand studies respiration in animals & man in prolonged hypoxia (*Acta Physiol Scand* 30/335, 343/54) . . . JR Bendall concludes myokinase is absorbed on muscle fibers in complex blocking contractile sites or catalyzing adenosine triphosphate in present of Mg (*Pros Roy Soc* B 142/409/54) . . . HOJ Collier analyses *Chemotherpy of Infections* (Wiley, NY, 54, 248pp, $4) . . . L Csalay & Harvath show detoxifying action of cortisone on histamine (*Acta Physiol Acad Sci Hung* 5/435/54) . . . M Dubuisson analyses *Muscular Contraction* (CC Thomas, Springfield, Ill, 54, 243pp, $6) . . . LF Fieser discusses biochemistry of cholesterol & its relation to cancer (*Science* 119/710/May 21/54) . . . L Geitler describes *Endomitose & Endomitotische Polyploidisierung* (Lange, Maxwell & Springer, NY, 22, 54, 120pp, $4.5) . . . DM Greenberg edits *Chemical Pathways of Metabolism*

(Academic Press, NY, 54, 2 vols, 1000pp, $27) . . . D Hattyasy shows persons free from dental caries have high saliva fluoride (*Acta Med Acad Sci Hung* 5/419/54) . . . AF Huxley & Co give structural interpretation of muscle contraction (*Nature* 173/971, 973/May 22/54) . . . HD Laszlo & PS Henshaw review plant materials used by primitive peoples to affect fertility (*Science* 119/626/May 7/54) . . . O Lindberg & L Ernster describe *Chemistry & Physiology of Mitochondria & Microsomes* (Lange, Maxwell & Springer, NY 22, 54, 160pp, $6.5) . . . CC Macklin suggests pneumocytes form pulmonary alveolar mucoid film (*Lancet* 266/1099/May 29/54) . . . J Mardones finds thiotic acid decreases alcohol intake on B deficiency (*Science* 119/735/May 21/54) . . . JR Robinson & Co find kidney slices produce ammonia in vitro (*J Physiol* 124/1, 8/54) . . . HS Simms & Co show cholesterol lowers antilipfanogen & liberates lipfanogen with deposition of fat (*J Gerontol* 9/133/54) . . . I Sperber analyses specificity of renal tubular transport mechanisms (*Arch Internat Pharmaco* 97/221/54) . . . H&M Staudinger discuss *Makromolecular Chemine & Ihre Bedeutung für die Protoplasmaforschung* (Lange, Maxwell & Springer, NY, 54, 80pp, $3.7) . . . T Valyi-Nagy & B Kelentei show adrenolytic action of malonic dinitrile (*Acta Physiol Acad Sci Hung* 5/495/54) . . . PR White describes *The Cultivation of Animal & Plant Cells* (Ronald Press, NY, 54, 210pp, $6) . . . FF Yonkman edits symposium on rauwolfia alkaloids (serpenia) in medicine (*Ann NY Acad Sci* 59/1–140/54) . . .

General: RT Abbott classifies *American Seashells* (D VanNostrand, NY, 54, 541pp, $12.5) . . . M Bonaparte & Co edit *Sigmund Freud's Letters* (Basic Books, NY, 54, 310pp, $6.75) . . . DL Farnsworth discourses on mental health in university life (*Ment Hyg* 38/34/54) . . . I Galdston gives *The Meaning of Social Medicine* (Harvard Univ Press, Cambridge, Mass, 54, 154pp, $2.75). AH Maslow notes instinctoid nature of basic needs (*J Personal* 22/326/54) . . .There's a Saturday Review Symposium *Is The Common Man Too Common?* (Univ Oklahoma Press, Norman, 54, 160pp, $2.75). WC Williams offers *The Desert Music & Other Poems* (Random, NY, 54, 90pp, $3) . . .

August, 1954

Hot Weather Amusement: OW Anderson makes *National Family Survey of Medical Costs & Voluntary Health Insurance* (Health Information Edition, NY, 54, 80pp) . . . K. E. Appel & M.M. Heyman recommend psychiatric social workers as aids in group process teaching (*J Med Edu* 29/38/July 54) . . . R Arnheim studies *Art & Visual Perception: A Psychology of the Creative Eye* (Univ California Press, Berkeley, 54, 420pp, $10) . . . WJ Beecker discourses on coriolis forces & bird migration (*Sci Month* 79/27/54) . . . A Bethe & H Netter give appreciation of Rudolf Hober's life (1873–1953), (*Pfluger's Arch ges Physiol* 259/1, 4–13/54) . . . L Charmichael cautions against laziness in scholarly life (*Sci Month* 78/208/54) . . . F Dessauer develops *Quantenbiologie: Einführung in einen neuen Wissenszweig* (Springer, Berlin, 54, 178 pp, DM 18.6) . . . CM Fleming compares general medical practice in America & Gt Britain (*Brit Med J* 1/1391/June 19/54) . . . JZ Fullmer relates contemporary science & poetry (*Science* 119/855/June 18/54) . . . C Hartshorne talks about mind matter & freedom (*Sci Month* 78/314/54) . . . Kentucky

symposes on *Learning Theory, Personality Theory & Clinical Research* (Wiley, NY, 54, 170pp, $3.5) . . . B Kisch recalls *Forgotten Leaders in Modern Medicine* (*Trans Am Philosoph Soc* 44/2/179pp/54) . . . G Mora well describes remarkable psychiatric contributions of Vincenzo Chiarugi (1759–1820) of Florence (*Bull Isaac Ray Med Lib, Providence* (2/51–104/54) . . . M Rokeach analyses nature of dogmatism (*Psych Rev* 61/194/54) . . . M Schneider eulogizes physiologist Hermann Rein (1898–1953) (*Pfluger's Arch ges Physiol* 259/14–20/54) . . . AL Strauss finds usual strain & harmony in American-Japanese war marriages (*Marr Fam Living* 16/99/54) . . . R Thauer describes hypoxic studies of Erich Opitz (1909–1953) (*Pfluger's Arch ges Physiol* 259/21–29/54 . . . US Atomic Energy Commission publishes *In the Matter of J Robert Oppenheimer: Texts of Principal Documents & Letters of Personnel Security Board May 27–June 29, 1954.* (US Gov't Printing Office, Washington, DC, 54, 67pp, 50c) . . . R Walker criticizes logic of parapsychologist JB (ESP) Rhine (*Sci Month* 79/1/54) . . . HV Warren discusses geology & health (*Sci Month* 78/339/54) . . .

PRECLINICAL: JM Barnes & FA Denz describe methods of estimating chronic toxicity (*Phramacol Rev* 6/191/54) . . . DK DeJongh finds no evidence that analgesic action of morphine is cholinergic (*Acta Physiol Pharmacol Neerland* 3/164/54) . . . P DeNicola offers *La Diagnosi dei Difetti di Coagulzione* (*Biblioteca Haematologica,* xvii, Pavia, Viscontea, 54, 273 pp) . . . E Florey finds inhibitory & excitatory factor for mammalian central nervous system (*Arch Internat Physiol* 62/33/54) . . . K Fukushima & Co describe function of tail of neutrophilic leukocytes (*Med J Osaka Univ* 5/57/54) . . . HR Hulpieu & Co find thiamin necessary for metabolism of acetaldehyde in stage two of alcohol metabolism (*Quart J Stud Alcohol* 15/189/54) . . . FH Johnson, H Eyring & MJ Polissar apply molecular theory to interpretation of biological reactions in *The Kinetic Basis of Molecular Biology* (Wiley, NY, 54, 756pp, $12.5). DA Karnofsky considers the discipline of cancer chemotherapy (*Geriatrics* 9/293/54) . . . S Koletsky & JH Christie find radioactive phosphorus carcinogenic in doses more than 1 microcure per gm body wt (*Proc Soc Exp Biol Med* 86/266/54) . . . HR Mahler & DE Green discuss metallo-flavoproteins & electron transport (*Science* 120/7/July 2/54). A Meister describes enzymatic transfer of alpha-amino groups (*ibid,* 43/July 9/54) . . . P Mitchell & J Moyle suggest lipo-glycerophosphate protein common factor in cell envelope of Gram positive organisms (*J gen Microbiol* 10/533/54) . . . J. W. Richardson finds neonatal anoxia reduces learning ability (*Proc Soc Exp Biol Med* 86/34/54). M Weatherall considers influence of drugs on porphyrin metabolism (*Pharmacol Rev* 6/133/54) . . . RH Wilson & AJ Cox, Jr, find no toxicity on long ingestion of food antioxidant conidendrol (*Proc Soc Exp Biol Med* 86/247/54) . . .

CLINICAL: GM Carstairs discusses cultural factors in choice of intoxicants, indicating alcohol as relief from aggressive drive & hashish as aid to contact with spiritual reality (*Quart J Stud Alcohol* 15/200/54) . . . DC Conrad studies factors in duration of psychotherapy (*J Clin Psych* 10/229/54) . . . T Johnson finds coxsackie virus type B3 as cause of Bornholm disease, epidemic pleurodynia (*Arch ges Virus Forsh* 5/401/54) . . . PE Klopsteg & PD Wilson

edit *Human Limbs & Their Substitutes* (McGraw-Hill, NY, 54, 714pp, $12) . . . AG Riddell & WW McDermott suggest ammonia intoxication in hepatic coma as basis for therapy (*Lancet* 266/1263/June 19/54) . . .

SEPTEMBER, 1954

AMBIVALENT WORD-COINING QUACKERY: A Massa is cloudily motivated with "lysembrio," an agent for rejuvenating treatment (*J Indian Med Prof* 1/144/54, a cheap sheet) . . . *New Series: Disease-A-Month* offers LA Rantz on rheumatic fever; RT Shank on hepatitis; RW Wilkins on hypertension; TA Warthin on gastric bleeding; MB Strauss on renal failure; E Jawetz on urinary tract infection; RW Rawson on thyrotoxicosis; AL Alexander on bronchial asthma; EA Stead on heart failure; AC Ernstene on coronary artery disease; M Miller on diabetes mellitus & MM Wintrobe on anemias (Year Book Publ, Chicago, 54, $8 annually).

LOYALTY, SECURITY, SCIENTIFIC IDEALS & MORALITY: B Bliven dramatizes *In the Matter of J Robert Oppenheimer* (US Govt Printing Office, 54, 992pp, $2.75): "A conflict to delight Shakespeare or the Greeks: let the hero be the creator, in some measure, of the restricted data; let him be a thoughtful man who has come by a hard route to agree fully that the national security outranks every other consideration; let him be the author, in part, of the very security system that becomes the weapon of his own downfall; let him be Everyman, but twice life size and infinitely more complicated; and finally let his character flaw, the fault that foredooms him, be loyalty to friends and an extravagant measure of human compassion" (*Sat Rev* Aug 21/54, p 19) . . . LB Loeb discusses security in a scientific age (*Science* 120/155/July 30/54) . . . W. Seifriz, in commenting on O Loewi's reflections on the study of physiology (*Physiol Rev* 16/1/54), calls for an intelligent biological system of ethics (*Science* 120/87/July 16/54). A Vallentin, in *The Drama of Albert Einstein* reveals a modern Socrates (Doubleday, NY, 54, 312pp, $4).

GENERAL: L Alexander draws lessons from suicide of Ludwig II of Bavaria in 1886 (*Am J Psychiat* 111/100/54 . . . L Festinger logically develops theory of social comparison processes on basis of drive for self-evaluation (*Human Rel* 7/117/54) . . . M Sandoz describes *The Buffalo Hunters* (Hastings, NY, 54, 372pp, $4.5) . . . GG Stern describes assessment of student personality structure (*J Pastoral Care* 8/76/54) . . . H Williams presents *Don Quixote of the Microscope: An Interpretation of the Spanish Savant Santiago Ramon y Cahal* (Jonathon Cape, London, 54, 235pp, 20s).

CLINICAL: T Brown & Co discuss psychologic factors in low back pain (*New Eng J Med* 251/123/July 22/54) . . . FB Byron describes pathogenesis of hypertensive changes in tissues (*Lancet* 2/202/July 31/54) . . . A Gigon edits symposium on thrombosis & emboli (*Schw Med Wchnschr* 84/773–838/July 17/54) . . . Our A Hansen, JG Sinclair & HF Wiese suggest dietary fat factor necessary for maturation of epithelial & sebaceous cells (*J Nutr* 52/541/54) . . . LS King raises pertinent question, What Is Disease? (*Phil Sci* 21/193/54) referring to E Bartlett's *An Essay on the Philosophy of Medical Science* (Lea & Blanchard, Phila, 1844, 310pp) . . . J LeBlanc

& Co find escorbic acid improves human resistance to cold (*Canad J Biochem Physiol* 32/407/54) . . . H Malmros & Co describe essential hyperlipemia (*Acta Med Scand* 149/91/54) . . . EK Marshall Jr & AH Owens conclude trichlorethanol is better hypnotic than chloral hydrate (*Bull Johns Hopkins Hosp* 95/1/54) . . . LF Peltier offers method of detecting fat emboli in circulating blood (*Surg* 36/198/54). E Robins & IN Mensh review validation in interrelations of biochemistry, psychiatry & psychology (*J Abn Soc Psychol* 49/435/54) . . . MH Seevers discusses adaptation to narcotics (*Fed Proc* 13/672/54) . . . AR Stone & Co recommend group psychodiagnosis as preliminary to effective group psychotherapy (*Internat J Group Psychother* 4/274/54). HR Viets reviews myasthenia gravis (*New Eng J Med* 251/97, 141/July 15, 22/54).

PRECLINICAL: EA Boell edits *Dynamics of Growth Processes* (Princeton Univ Press, 54, 312pp, $7.5). RM Brickner labels neural fractionating & combining system & discusses appetitive behavior & sign stimuli (*Arch Neurol Psych* 72/1, 92/54) . . .HA Krebs in Herter Lectures discusses biosynthesis, adenosine metabolism & teleology (*Bull Johns Hopkins Hosp* 95/19, 34, 45/54). R Peters analyses action of ancient poison fluoroacetate (*Endeavor* 13/147/54). Rockefeller Institute announces *J Biophys & Biochem Cytology*, Vol 1 No. 1, Jan 55, $7.50. A Stoll well reviews Cordiotonics from *Scilla maritima* (*Experientia* 10/282/July 15/54). E Tralucchi & A Baserga review antimitotic agents (*Gior Ital Chemiother* 1/1, 15/54).

OCTOBER, 1954

GENERAL: ED Adrian discusses science & human nature (*Nature* 174/433/Sept 4/54). E Brodman describes *Development of Medical Bibliography* (Med Libr Asso, 54, 226pp, $5). JE Dunphy defines the university surgeon (*Surg* 36/353/54). Educational issue *Brit Med J* (2/479–514/Aug 28/54) contains symposium on selection of medical students from various countries, RW Parnell's study of physique & performance, JM Stalker's plea for hospital technique in the home, & J Trueta's appraisal of scientific achievements of Michael Servetus (1511–1553). PG Frank opens symposium on reasons for acceptance of scientific theories, with B Moore noting influence of political creeds, CW Churchman proposing pragmatic theory of induction, & R Rudner remarking on value judgments (*Sci Month* 79/139–153/54). RM Goldenson steps into the world of insane (*Look* Sept 21/54). CJ Herrick salutes 100 volumes of *J Comp Neurol*, founded by brother CL in 1891 (100/717/54). W Jackson edits *Communication Theory* (Academic Press, NY, 54, 544pp, $11). L Lasagna & JM von Felsinger criticize volunteer subjects in research (*Science* 120/359/Sept 3/54). H. Lehmann gives fascinating account of distribution of sickle cell gene (*Eugenics Rev* 46/121/54). RH Major offers great *History of Medicine* (CC Thomas, Springfield, Ill, 54, 2 vols, 1200pp, $14.50). A Rapoport suggests *Operational Philosophy: Integrating Knowledge & Action* (Harper, NY, 54, 269 pp, $3.75). MM Roberts gives *American Nursing: History & Interpretation* (Macmillan, NY, 54, 688pp, $6). BR Sappenfield offers *Personality Dynamics: An Integrative Psychology of Adjustment* (AA Knopf, NY, 54, 428pp, $5.5). JD Williams

writes *The Compleat Strategyst* (McGraw-Hill, NY, 54, 300pp, $4.75).

PRECLINICAL: HT Clarke edits *Ion Transport across Membranes* (Academic Press, NY, 54, 310pp, $7.5). J Crossland & AJ Merrick show anesthesia increases brain acetylcholine (*J Physiol* 125/56/54). HE Hoff & CG Breckenridge describe intrinsic mechanisms in periodic breathing (*Arch Neurol Psychiat* 72/11/54). F Huidebro confirms pharmacology of chlordimethylamine-propyl-phenothiazine (chlorpormazine) as similar to Dibenamine (*Arch Internat Pharmaco* 98/308/54 as reported by S Courvoisier & Co. (*ibid* 92/305/53). L Pillemer & Co note properdin-protein system in sera as factor in immunity (*Science* 120/279/Aug 20/54). DH Wenrich & Co edit *Sex in Microorganisms* (AAAS, Washington, DC, 54, 362pp, $5.75). JC White & GH Beaven review varieties of human hemoglobin in health & disease (*J Clin Path* 7175/54). H Winterstein shows hyperthermia tends toward alkalosis, hypothermia toward acidosis (*Arch Exp Path Pharmakel* 223/1/54).

MEDICAL PSYCHOLOGY: J Abrahams & E Varon relate *Maternal Dependence & Schizophrenia* (International Univ Press, NY, 54, 240pp, $4). L Bender offers *A Dynamic Psychopathology of Childhood* (CC Thomas, Springfield, Ill, 54, 296pp, $7.5). H Gastaut gives *The Pilepsies: Electroclinical Correlations* (Thomas, Springfield, Ill, 54, 176pp, $4.75). P Greenacre edits *Affective Disorders* (Internat Univ Press, NY, 54, 212pp, $3). RG Heath edits *Tulane Psychiatrists' Studies in Schizophrenia: A Multidisciplinary Approach to Mind-Brain Relationships* (Harvard Univ Press, Cambridge, Mass, 54, 623pp, $8.5). PH Hoch & J Zubin edit *Depression* (Grune & Stratton, NY, 54, 287pp, $5.5). JQ Holsopple & FR Miale offer *Sentence Completion: A Projective Method for the Study of Personality* (CC Thomas, Springfield, Ill, 54, 192pp, $5.5). SB Kutash & RH Gehl describe *The Graphometer Projection Technique: Clinical Use & Standardization* (CC Thomas, Springfield, Ill, 54, 148pp, $3.75). S Livingston describes *The Diagnosis & Treatment of Convulsive Disorders in Children* (Thomas, Springfield, Ill, 54, 320pp, $9.5). LR Wolberg reveals *The Technique of Psychotherapy* (Grune & Stratton, NY, 54, 883pp, $14.75). J Wolke claims reciprocal inhibition as main basis of psychotherapeutic effects (*J Neurol Psychiat* 72/205/54).

CLINICAL: I Aird & Co show blood group O tends toward peptic ulcer & LA Pike & Co toward toxemia of pregnancy (*Brit Med J* 2/315, 321/Aug 7/54). DW Terry & Co describe homozygous hemoglobin C: a new hereditary hemolytic disease (New Eng J Med 251/365/Sept 2/54).

NOVEMBER, 1954

CLINICAL: MD Altschule reviews physiologic psychology of neurosis (*New Eng J Med* 251/476/Sept 16/54) . . . P Balint & Co analyse post-hemmorrhagic anuria (*Acta Physiol Acad Sci Hungar* 6/57, 69, 81/54) . . . W Bartschi-Rochaix considers relation of migraine to epilepsy (*Schw Med Wchnschr* 84/1139/Oct 2/54) . . . British Postgraduate Medical Federation, University of London offers *Lectures on the Scientific Basis of Medicine*, Vol I, 18 Lectures; Vol II, 19 Lectures, obtainable from Jon de Graff, 64 W

23, NY10 . . . K Bronsch notes thyroxin action in ricketts (*Ztschr ges Exp Med* 124/118/54) . . . LM Cassinrelli discusses influence of acclimatization to altitude on fertility (*Anal Facul Med Univ Nac May San Marcos Lima* 36/419/54) . . . AC Corcoran & Co review handling of hypertension (*Am J Med* 17/383/54 . . . TM French offers 2 vols on *The Integration of Behavior* (Univ Chicago Press, 54, 231 & 331pp, $11.5) . . . JA Gengerelli & FJ Kirkner edit *Psychological Variables in Human Cancer* (Univ California Press, Berkeley, 54, 141pp, $3) . . . WS Hoffman describes *The Biochemistry of Clinical Medicine* (Year Book, Chicago, 54, 681pp, $12) . . . A Hottinger & F Hauser edit *Modern Problems in Pediatrics* (Vol I, S Karger, NY, 54, 844pp, $14) . . . B Kissin & N Kruger note high serum citrate in Paget's disease (*Am J Med Sci* 228/301/54) . . . TL Peele gives *The Neuroanatomical Basis for Clinical Neurology* (McGraw-Hill, NY, 54, 574pp, $12.5) . . . O Refvem reviews pathogenesis of sarcoidosis (*Acta Med Scand* 149/Suppl 294/Oslo, 54, 146pp) . . . Wm Requarth outlines *Diagnosis of Acute Abdominal Pain* (Year Book, Chicago, 54, 243pp, $5) . . . H Statland discusses *Fluid & Electrolytes in Practice* (Lippincott, Phila, 54, 195pp, $5) . . . B Wolstein considers psycholytic problem of *Transference* (Grune & Stratton, NY, 54, 220pp, $5).

PRECLINICAL: BE Abreu & Co show protoveratrine A has greatest hypotensive & emetic action via vagi & carotid sinus (*J Pharmacol* 112/73/54) . . . J Boss & JH Green show nerve connections in baroceptor areas of carotids (*J Physiol* 124/43P/54) . . . E Boyland analyses mutagens (*Pharmacol Rev* 6/345/54) . . . F Brink describes role of calcium ions in neural processes (*ibid* 9, 243) . . .HJ Cruft & Co show abnormal histones from malignant cells (*Nature* 174/580/Sept 25/54) . . . M Delbruck suggests path of replication of desoxyribonucleic acid with significance for genetics (*Proc Nat Acad Sci* 40/783/54) . . . VA Drill edits huge *Pharmacology in Medicine* (McGraw-Hill, NY, 54, 1264pp, $19.5) . . . WF Floyd & AT Welford edit *Fatigue and Human Factors in Equipment Design* (De Graff, NY, 54, 196pp–$4; 132pp, $4) . . . T Fukuoka finds bile salt effects on renal function (*Hiroshima J Med Sco* (2/365, 377, 383, 389/54) . . . JS Gravestein & Co study antitussive agents (*J Applied Physiol* 7/119/54) . . . CA Handley & JH Moyer say sodium & water excretion altered by renal hemodynamics but not by direct effect of sympathetics on renal tubules (*J Pharmacol* 112/1/54) . . . I Huszak & Co show serum & heparin compete in hyaluronidase system (*Acta Physiol Acad Sci Hungar* 6/1/54) . . . B Minz discusses *The Role of Humoral Agents in Nervous Activity* (CC Thomas, Springfield, Ill, 54, 156 pp, $5) . . . RW Moncrieff characterizes odours by adsorption properties related to smell & not to chemical constitution (*J Physiol* 125/453/54) . . . S Rothman discusses *Physiology & Biochemistry of the Skin* (Univ Chicago Press, 54, 741pp, $19.5) . . . MA Slusher & S Roberts fractionate hypothalamic tissue for pituitary stimulants & find lipoprotein fraction releasing ACTH (*Endocrinol* 55/245/54) . . . JM Steele edits vol 6 *Methods of Medical Research* (Year Book, Chicago, 54, 271pp, $7) . . . H Tabor reviews metabolism of imidazoles (*Pharmacol Rev* 6/299/54) . . . CA Winter & L Flataker find codeine, narcotine, propadrine & nalorphine useful anti-

tussive agents (*J Pharmacol* 112/99/54).

GENERAL: W Bremberg gives *Man Above Humanity* (Lippincott, Phila, 54, 218pp, $3.5) . . . Note Editorial review of Oppenheimer case (*Nature* 174/569/Sept 25/54) . . . RS Brumbaugh reviews Plate's genetic theories (*J Heredity* 45/191/54) . . . HJ Goldings studies avowal & projection of happiness (*J Personality* 23/30/54) . . . N Kelman & Co contribute well to memory of Karen Horney (1885–1952) in medium she founded (*Am J Psychoanaly* 14/1–122/54) . . . S Lilly edits symposium on Social History of Science (*Centaurus* 3/1–182/53) . . . M Morgenan opens symposium on "Operationalism" (*Sci Month* 79/209–231/54) . . . HS Perry illustrates selective inattention by public attitudes toward A bomb (*Psychiat* 17/225/54) . . . G Sarton gives Montgomery Lectures on *Ancient Science & Modern Civilization* (Univ Nebraska Press, Lincoln, 54, 134pp, $2.15) . . . DW Singer well associates science & confidence in the rational mind (*Centaurus* 3/166/53).

DECEMBER, 1954

BROWNINGS: M Bishop offers *A Bowl of Bishop: Museum Thoughts & Other Verses* (Dial, NY, 54, 106pp, $3) . . . EG Boring notes psychological factors in the scientific process (*Amer Sci* 42/639/54) EW Chase & daughter Ilka are *Always in Vogue* (Doubleday, NY, 54, 381pp, $5) . . . A Cooke gives *A Commencement Address* & CS Coon *The Story of Man* (Knopf, NY, 54, 30 & 212pp, $1 & $6.75) . . . M Covarrubias adds *The Eagle, The Jaguar & The Serpent* (Knopf, NY, 54, 320pp, $15) . . . A Dunhill mellows *The Gentle Art of Smoking* (Putnam, NY, 241pp, $3.75) . . . B Evans laughs at *The Spoor of Spooks & Other Nonsense* (Knopf, NY, 54, 258pp, $4.5) . . . A must is J Fletcher's wise *Morals and Medicine* (Princeton Univ Press, 54, 252pp, $4.5) . . . E Frenkel-Brunswick, BF Skinner, J Richfield & M Scriven sympose on psychoanalysis & the scientific method (*Sci Month* 79/293–310/54) . . . L Fermi finds *Atoms in the Family* (Univ Chicago Press, 54, 267pp, $4) . . . V Gildersleeve puts up *Many a Good Crusade* (Macmillan, NY, 54, 434pp, $4) . . . G Highest likes to be *A Clerk of Oxenford* (Oxford Univ Press, NY, 54, 272pp, $4) . . . E Kilman & T Wright puff *Hugh Roy Cullen: Story of American Opportunity* (Prentice-Hall, NY, 54, 432pp, $4) . . . JP Marquand covers *Thirty Years* of short stories (Little Brown, Boston, 54, 466pp, $5) . . . Pupils of Wm Pepperell Montague (1873–1953) offer tributes (*J Philosophy* 51/593–636/Oct 14/54) . . . D McCord laughs at *Odds Without Ends* (Little Brown, Boston, 54, 139pp, $3) . . . CR Rogers & M Tummi discuss theory of & obstacles to creativity (*Etc* 11/249, 261/54) . . . WH Schneider draws well on "Danger: Men Talking" (*ibid* p 243) . . . J Shepley & C Blair are no help in *The Hydrogen Bomb* (McKay, Phila, 54, 244pp, $3) . . . J Shercliff whirls with *Jane Avril of the Moulin Rouge* (Macrae Smith, Phila, 54, 244pp, $3.5) . . . FGW Smith & Chapin look to *The Sun, the Sea and Tomorrow* (Scribner's, NY, 54, 210pp, $3.5) . . . AB Toklas purees a *Cook-Book* (Harper, NY, 54, 288pp, $4) . . . JR Ullman likes *The Age of Mountaineering* (Lippincott, Phila, 54, 352pp, $6) . . . S Waksman describes *My Life with the Microbes* (Simon & Schuster, NY, 54, 346pp, $5) . . . HA Witkin & Co, with G Murphy as editor, study *Personality*

through Perception (Harper, NY, 54, 592 pp, $7.5) . . . WC Williams gives his *Collected Essays* (Random House, NY, 54, 312pp, $4.5) . . . G Zilboorg discusses *The Psychology of the Criminal Act and Punishment* (Harcourt Brace, NY, 54, 150pp, $3.5).

CLINICAL: GE Cartwright & Co consider Wilson's disease (hepatolenticular degeneration) due to copper accumulation (*J Clin Invest* 33/1487/54) . . . W Dameshek describes hemotoxic drug reactions (*Post-Grad Med* 16/369/54) . . . G Enhorning & B Westin show oxygenated heparin blood into umbilical vein may save asphyxiated new-borns (*Acta Physiol Scand* 31/359/54) . . . GS Jones covers *Management of Endocrine Disorders of Menstruation & Fertility* (CC Thomas, Springfield, Ill, 54, 198pp, $5) . . . H McLorinan discusses changes in infectivity of infectious diseases (*Med J Austral* 2/533/Oct 2/54) . . . AE Mourant describes *The Distribution of the Human Blood Groups* (Blackwell, Oxford, 54, 438pp, $6) . . . JP Murphy discusses *Cerebrovascular Disease* (Year Book, Chicago, 54, 408pp, $12) . . . JM Sheldon & Co review urticaria (*J Allergy* 25/525/54) . . . MB Sulzberger & F Herrmann note *The Clinical Significance of Disturbances in the Delivery of Sweat* (CC Thomas, Springfield, Ill, 54, 222pp, $5) . . . MA Wessel & Co survey paroxysmal fussing ("Colic") in infants (*Pediatrics* 14/421/54).

PRECLINICAL: G Asboe-Hansen edits *Connective Tissue in Health & Disease* (Munksgaard, Copenhagen, 54, 432pp, $7.5) . . . AC Burton notes relation of structure to function in blood vessels walls (*Physiol Rev* 34/619/54) . . . A Carlson & Co study biological effects of vitamin D (*Acta Physiol Scand* 31/301–317/54) . . . AL Charnoff & Co report Hemoglobin E, hereditary anomaly (*Science* 120/605/Oct 15/54) . . . HG Davies & Co use interference microscopy for quantitative histochemical estimation (*Quart J Micros Sci* 95/271/54). FJ Dixon studies metabolism of antigen & antibody (*J Allergy* 25/487/54) . . . RW Gerard reports experiments in microrevolution (*Science* 120/727/Nov 5/54) . . . K Inoue & Co show stimulation of ventromedial hypothalamic nucleus is followed by fall in blood cholesterol (*Med J Osaka Univ* 5/475/54) . . . GW Kidder edits symposium on lipoic (thioctic) acid (*Fed Proc* 13/695-743/54) . . . AM Lassek gives *The Pyramidal Tract: Its Status in Medicine* (CC Thomas, Springfield, Ill, 54, 166pp, $4) . . . S Margolin & Co report anticardiac action of steroid alkamines (*Science* 120/576/Oct 2/54) . . . PD Orahovats & Co show undesired actions of morphine are reduced by nalorphine (*J Pharmacol* 112/246/54) . . . FE Russell & A Van Harroveld note heart inhibition from venom of sting-ray, "Urobatis Halleri" (*Arch Internat Physiol* 62/322/54) . . . WH Seegers opens symposium on prothrombin & fibrinogen (*Physiol Rev* 34/711–760/54).

TEXAS REPORTS ON BIOLOGY AND MEDICINE

Volume 12, 1954

Author Index

	Page
Anigstein, Ludwik	3, 945, 1047
Awapara, Jorge	799
Bailey, Herbert A.	110
Bang, Frederik B.	805
Barnes, William	3, 945
Beerstecher, Ernest, Jr.	207, 212
Benedict, Albert A.	21
Berry, George P.	575
Blocker, T. G., Jr.	354, 509
Borghese, E.	215
Brown, T. McB.	577
Brueckner, Arthur L.	174, 178, 524, 1061, 1067
Brues, Austin M.	931
Burdette, Robin I.	138
Burlage, Henry M.	229, 500, 551
Bussell, Robert	336
Cameron, L. E.	438
Cardo, LaVerne	207
Childers, John H.	979
Clark, R. Lee, Jr.	30
Cobb, Beatrix	30, 738
Cook, Sue R.	1067
Cornyn, John	207, 212
Davison, W. C.	655
de Chenar, C.	453
Delaha, Edward C.	178, 524, 1061, 1067
Dufrenoy, Jean	145
Eads, R. B.	489
Elkins, Joe C.	39
Engley, Frank B., Jr.	21, 49, 64, 236, 250
Felton, Harriet M.	960
Flowerman, Samuel H.	260
Fremont-Smith, Frank	568
Futch, . D. III	423
Gaggero, Aldo	960

	Page
Garrett, James F.	634
Gerstner, Herbert B.	383
Gey, George O.	805
Gey, Margaret K.	805
Gibson, Susan M.	229
Giglio, Frank A.	278
Gilliland, P. F.	326, 345
Goolishian, H. A.	972
Gonzales, Federico	828
Goth, Andres	110
Green, David E.	284
Gregory, R. L.	423
Grimes, J. E.	489
Haedicke, Thomas A.	975
Ham, George C.	665
Handley, Carroll A.	464
Hansen, Arild E.	67
Harper, Rita	207
Harris, Titus	305
Harrower, M. R.	72, 561, 766
Hayden, K.	470
Hild, Walther	474
Hinds, E. C.	86
Holbrook, W. P.	760
Holsopple, J. Q.	648
Howe, C. D.	30
Hsu, T. C.	833
Huggins, R. A.	464
Huston, Paul S.	637
Irons, J. V.	489
Jacobson, Carlyle	672
Jirgensons, B.	799
Johnson, Joseph K.	979
Jones, Bobby	975
Junqueira, L. C.	94
Kirchoff, Helen	292
Kline, Ira	865
Kobayashi, M.	423
Koffka, Kurt	98
Kohn, Lawrance A.	662
Korenchevsky, V.	1006
Kubie, Lawrence S.	608, 692, 742
Kuzell, Charles	588

	Page
Lackey, Robert W.	110
Lange, Robert D.	887
Lara, Jorge Y.	300
Lea, Royal B.	278
Leake, Chauncey D.	627, 632, 775
Lee, Russell V.	584
Leighton, Joseph	847, 865
Lipschultz, A.	115
Llamon, W. T.	653
Lockingen, L. S.	196
Marcus, Peter M.	126, 874
May, L. G.	423
May, Rollo	622
McConnell, Kenneth P.	945
McDanald, Eugene	619, 772
McKenna, George F.	229, 500, 551
McSwain, Barton	916
Mendel, L.	429
Meyers, Russell	613, 778
Mitis, Z. C.	305
Moller, Knud O.	313
Moloney, William C.	887
Morlan, Harvey B.	10377
Mullins, J. Fred	1047
Murray, Margaret R.	898
Nakai, J.	345, 354, 509
Nowinski, W. W.	305
Nelson, Helen E.	1067
Nilasena, Samchai	1047
Orr, Mary Faith	916
Page, Carl F.	250, 278
Palmer, Eddy D.	174, 178, 1061, 1067
Pappas, Peter	945
Peck, Harris B.	744
Petermann, Mary L.	921
Pike, Robert M.	138
Pollard, Morris	21, 336, 341
Pomerat, C. M.	326, 345, 354, 470, 509, 960
Portman, Oscar W.	945
Pratt, Robertson	145
Prince, Frank M.	370, 1037
Rabinovitch, M.	94
Reagan, Reginald L.	174, 178, 524, 1061, 1067

	Page
Richey, Everett O.	383
Rigdon, R. H.	182, 292
Robbins, C. L.	586
Rose, George	1074
Rottersman, W.	640
Russell, Glenn V.	528
Sanger, W. T.	582
Seidenfeld, M. A.	605, 645
Selye, Hans	390
Seybold, W. D.	86
Scott, Martha Jordan	236, 250
Shorr, Ephraim	599
Smith, Leland L.	543
Smith, McDonald	470
Snyder, Clifford C.	336, 341
States, Sally J.	543
Stewart, Mildred T.	174, 178, 524, 1061
Stokes, Dorys-Mary	551
Stout, Arthur Purdy	898
Stroud, Agnes N.	931
Sulkin, S. Edward	138
Sullivan, Thelma	489
Sullivan, Thelma	489
Taylor, Alfred	229, 500, 551
Thomsen, M.	182
Tips, Robert L.	187
Trunnell, J. B.	30
Tsai, S. Y.	423
Volkmann, Carol	207, 212
Walker, S. C.	86
Watson, Robert	659
Watts, Fenwick L.	1047
Whitney, Dorothy M.	3, 945
Williams, Marjorie J.	429
Wingo, W. J.	196, 438
Wright, Beatrice	610
Wright, M. Erik	624

TEXAS REPORTS ON BIOLOGY AND MEDICINE

Volume 12, 1954

Subject Index

	Page
Acid phosphatase content of leukocytes in hematological disorders	429
Adenocarcinoma of the parotid gland	847
Adenocarcinoma of the pancreas	847
Ageing, processes of	1006
Alkaline content of leukocytes in hematological disorders	429
American Harpactorine genus	39
Antibacterial action of normal blood, effect of gastric mucin on	236
Antibiotics in the treatment of wounds	145
Antirickettsial homologous gamma globulin	3
Antitumoural autodefense	115
Application of neotrazolium to outgrowths of human neoplasms in tissue culture	126
Autointoxications and processes of ageing	1006
Bacteriological problems in maintaining the chronic nephrectomized dog	278
Barbituric acid poisoning, treatment of	313
Bence-Jones proteins of multiple myeloma	799
Biochemical studies on the granulocytes in early leukemia	887
Blood cultures, gastric mucin as an adjuvant in	250
Carbarsone, response of trichomonas hominis to	975
Carcinoma, invasion of connective tissue by	865
Cardiovascular effects from a series of N-substituted arterenol derivatives compared with isoproylarterenol	464
Care of the chronically ill, medical and psychological teamwork in	561
Calling Attention To	200, 444, 558, 1084
Cancer case, treatment of	341
Cerebrospinal fluid analysis	453
Chemotherapy experiments with plant extracts and transplantable tumors	500
Cholesterol-C^{14}, paper chromatographic investigation of	543
Cholesterol-free diet on serum cholesterol in dogs	423
Chronically ill, medical and psychological team work in the care of	561
Citellus tridemlineatus tridecemlineatus	187
Common cold, electron microscopic studies of	174
Conference on medico-psychological teamwork	561
Copper sulfate	453
Cultured strains of normal and malignant cells of animals and man	805
Current status of rabies in Texas	489
Cytologic and biochemical studies on the granulocytes in early leukemia	887
Cytological studies on HeLa	833

PAGE

Disease, nature of 390

Effect of cholesterol-free diet on serum cholesterol in dogs..... 423
Effect of heat and time of exposure on human epidermis and cells from the skin and leg muscle of chick embryos..... 509
Effect of viruses on human neoplasms in tissue culture..... 336
Egg cultivated viruses, plant extracts tested against..... 551
Electron microscopic studies of an egg-adapted virus isolated from a patient with a common cold..... 1067
Electron microscopic studies of Erythrocytes from a patient affected with Hodgkin's Disease 178
Electron microscopic studies of a virus isolated from a patient with a common cold 174
Embryonic chick thyroid in tissue culture..... 828
Endocrinological observations in tissue cultures of posterior pituitary of dog and rat 474
Enzyme systems 284
Epidermoid carcinoma of the uterine cervix..... 847
Excretory duct ligature in the rat submaxillary gland..... 94

Gastric mucin, studies on.....49, 64, 236, 250
Gey's balanced salt solution versus protein containing fluids as freezing media 326
Granulocytes in early leukemia..... 887

HeLa, cytological studies on..... 833
Hematological disorders, alkaline and acid phosphatase content of leukocytes in 429
Hemiptera, Reduviidae 39
Hemophilus ertussis 969
Hodgkin's Disease 178, 1061
Histological observations in tissue cultures of osterior ituitary..... 474
Human breast carcinoma 916
Human cancer, studies on.....847, 865

Imrovements in letter styles for sight-testing charts..... 370
Infrared absortion studies 21
Intensity-time factor 345
Invertebrate nutrition 207, 212
In vivo and *in vitro* studies with Shigella dysenteriae..... 49
Isoroylarterenol 464

Letter styles for sight--testing charts..... 370
Leukemia, cytologic and biochemical studies on the granulocytes in..... 887

Macromolecular nucleorotein articles..... 921
Malignant melanoma, growth atterns..... 847
Mammalian hyothalamic nuclei cultivated "in vitro"..... 215

	Page
Man, thalamo-cortical relationshis in	528
Marital maladjustment, sychological factors in	72
Measurement of psychological factors in marital maladjustment	72
Medical and psychological team work in the care of the chronically ill	561
Medical psychology, a new profession	561
Mitochondrial cortex as seen in tissue cultures of nerve fibers	470
Multiple myeloma, Bence-Jones proteins of	799
Nature of diseases	390
Neck, teratoma of	86
Neotetrazolium to outgrowths of human neoplasms in tissue culture, application of	126
Normal breast epithelium	916
N-substituted arterenol derivatives	464
Nutrition, invertebrate	207, 212
Oniscus asellus	207, 212
On the nature of diseases	390
Organized enzyme systems	284
Paper chromatographic investigation of cholesterol-C^{14} and radioactive contaminants	543
Papillary adenocarcinoma of the thyroid gland	847
Pentobarbital poisoning	110
Peritoneal lavage in treatment of experimental pentobarbital poisoning	110
Personality, psychotherrapeutic adequacy and judgment of	972
Place of a psychologist among scientists	98
Plant extracts, chemotherapy experiments with	500
Plant extracts tested against egg cultivated viruses	551
Post-anaesthetic period of partuition	67
Posterior pituitary, histological and endocrinological observations in tissue cultures of	474
Precipitin antibodies produced against atropic agents	187
Primary disease processes observed at necropsy in pediatric cases	182
Proceedings, Texas Branch of the Society of American Bacteriologists, spring meeting, 1953	162
Proceedings, Texas Branch of the Society of American Bacteriologists, fall meeting, 1953	169
Proceedings, Texas Branch of the Society of American Bacteriologists, spring meeting, 1954	1052
Processes of ageing	1006
Psychological team work in the care of the chronically ill	561
Psychology program in a cancer research hosital	30
Psychotherapeutic adequacy and judgment of personality	972
Psychotherapy, myths in	260
Rabies, current status of in Texas	489
Radioactive contaminants, paper chromatographic investigation of	543

	Page
Radiation effects in tissue culture	931
Radiobiological research	383
Radioiodinated anti-organ antibodies	945
Radioiodinated antirickettsial homologous gamma globulin	3
Reactions of cells in tissue culture to hemophilus pertussis	960
Renal effects from a series of N-substituted arterenol derivatives compared with isopropylarterenol	464
Response of the Syrian hamster to 11 tropical viruses	524
Response of trichomonas hominis to carbarsone	975
Rhadinopsylla multidenticulatus	1037
Rheumatoid arthritis, serological reactions in	138
Salivary glands, tumors of	979
Sanguinin	1047
Secondary growth in tetrahymena geleii (Y)	196
Separable and multipurpose tissue culture chamber	1074
Serological reactions in rheumatoid arthritis	138
Shigella dysenteriae, *in vivo* and *in vitro* studies on	49
Sight-testing charts	370
Siphonaptera hystrichopsyllidae	1037
Skin reactions produced against atrophic agents	187
Small motion transducer for radiobiological research	383
Smoking habits of college students in Texas	292
Sponge matrix tissue culture	847, 865
Spontaneous pseudomonas infection in mice	64
Steroid therapy, therapeutic considerations	300
Studies on gastric mucin	236, 250
Studies on toxic plants for antibacterial effects, II	229
Subfamily Rhadinopsyllinae Wagner 1930, notes on	1037
Submaxillary gland	94
Team work in the care of the chronically ill	561
Teratoma of the neck	86
Tetrahymena Geleii (Y)	196, 438
Tetrazolium salts for short term tissue cultures of surgical specimens, use of	874
Texas Branch of the Society of American Bacteriologists, proceedings of, spring, 1953	162
Texas Branch of the Society of American Bacteriologists, proceedings of, fall, 1953	169
Texas Branch of the Society of American Bacteriologists, proceedings of, spring, 1954	1052
Texas, current status of rabies in	489
Therapeutic considerations in steroid therapy	300
Thalamo-cortical relationships in man	528
Thermal stress and tissue culture	326, 345, 354, 509

	Page
Tissue culture chamber	1074
Tissue cultures, effect of viruses on human neoplasms in	336
Tissue culture, embryonic chick thyroid in	828
Tissue culture of human breast carcinoma and normal breast epithelium	916
Tissue culture of nerve fibers, mitochondrial cortex as seen in	470
Tissue cultures of posterior pituitary	474
Tissue culture, reactions of cells in	960
Toxic plants for antibacterial effects	229
Transplantable tumors, chemotherapy experiments with	500
Treatment of a human cancer case with viruses	341
Trichomonas hominis to carbarsone, response of	975
Tumors of the salivary glands	979
Virus-like agent isolated from a patient affected with Hodgkin's Disease	1061
Virus preparations, infrared absorption studies of	21
Viruses, treatment of a human cancer case with	341
Wound healing effect of Sanguinin	1047